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GEORGE WILLIAMS HOOPER FOUNDATION FOR MEDICAL RESEARCH

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OF
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FOR MEDICAL RESEARCH

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THE CHEMOTHERAPEUTICS OF THE CHAULMOOGRIC ACID SERIES AND OTHER FATTY ACIDS IN LEPROSY AND TUBERCULOSIS

I. BACTERICIDAL ACTION; ACTIVE PRINCIPLE; SPECIFICITY

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Chaulmoogra oil has an empiric reputation in the treatment of leprosy that extends back into antiquity. This reputation is supported by the experience of modern leperologists. The old and less effective method of oral administration of chaulmoogra oil has been replaced, first by subcutaneous and intramuscular injection of oil mixtures, and more recently by intravenous injection of salts of the chaulmoogric acids; and investigators almost unanimously agree that arrest of the disease, improvement, and frequently cure follows adequate treatment with chaulmoogra oil and its derivatives. The importance of the fatty acid therapy of diseases due to the acid-fast group of bacteria has been greatly increased by the report of Rogers¹ on the intravenous use of "sodium morrhuate," the sodium salts of the fatty acids of cod-liver oil, in the treatment of tuberculosis. Rogers maintains that there is nothing absolutely specific in the products of chaulmoogra oil for leprosy, but that the unsaturated fatty acids of both chaulmoogra and cod-liver oils, and by implication the unsaturated fatty acids of any oil, are equally efficacious in either leprosy or tuberculosis. He believes that the unsaturated fatty acids act in some way on the fatty coating of acid-fast bacilli, presumably by injuring the capsule of the bacillus and exposing it to the destructive action of the tissues.

These therapeutic claims for the chaulmoogrates and morrhuates, if substantiated, would be of the greatest importance to medical science and human welfare. Further investigation is necessary, however, not only to confirm these claims, but also to discover the method of action of chaulmoogra and cod-liver oils, to identify and isolate the therapeutically active principle, if such exists, to determine its distribution in

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¹ Brit. Med. Jour., 1919, 1, p. 147; Indian Med. Gaz., 1919, 54, p. 165.

vegetable and animal oils, and to test the specificity of its action in leprosy, tuberculosis and other infectious diseases. Such an investigation conducted in the test tube, where the chemical and bactericidal aspects of the subject can be studied, and in animals in which the experiments can be adequately controlled, will, we believe, supply more precise information on at least some of the problems involved than would clinical observation alone. The scope of the experimental investigation which we have undertaken is indicated in the outline:

1. What is the method of therapeutic action, if any, of chaulmoogra oil in leprosy:
 - A. Direct or bactericidal
 - B. Indirect or physiologic
 - a. Stimulation of nonspecific lipolytic activity of the tissues which might attack the fatty capsule of acid-fast bacilli
 - b. Antigen for the production of more or less specific fat antibodies (Deycke,² Kleinschmidt,³ Lucke,⁴ Warden,⁵ Young⁶)
 - c. Disturbance of the ferment-antiferment balance of the body fluids (Jobling and Petersen⁷)
 - d. Production of lymphocytosis, which might act as intermediaries in the defense of the host
 - e. Nutritional only
 - C. Inactive?
2. What is the active therapeutic principle of chaulmoogra oil:
 - a. Chaulmoogric acid
 - b. Hydnocarpic acid
 - c. Palmitic acid
 - d. Glycerol or other alcohols
 - e. Gynocardin
 - f. Unidentified substance?
3. Is the therapeutic action of chaulmoogra oil specific for:
 - a. Leprosy
 Or does its action extend to:
 - b. Tuberculosis and other infections with acid-fast bacilli
 - c. Infections with nonacid-fast bacteria
4. Is the therapeutically active principle peculiar to:
 - a. Chaulmoogra oil
 Or is it found in:
 - b. Cod-liver oil (Rogers¹)
 - c. Other oils
 - d. Nonfatty substances

DEVELOPMENT OF THE FATTY ACID THERAPY

Chaulmoogra oil was formerly administered as whole oil by mouth in the empirical treatment of leprosy, often accompanied by local applications of the oil to the skin lesions or combined with other treatment. The nauseating

² Deutsch. med. Wchnschr., 1907, 33, p. 89.

³ Berl. klin. Wchnschr., 1910, 47, p. 57.

⁴ Jour. Immunol., 1916, 1, p. 456.

⁵ Jour. Infect. Dis., 1918, 23, p. 504.

⁶ Jour. Path. & Bacteriol., 1919, 22, p. 224.

⁷ Jour. Exper. Med., 1914, 19, p. 239.

property of this oil and the long course of treatment necessary to obtain therapeutic results seriously interfered with its efficient use. Nevertheless, improvement and even cure of leprosy attributed to chaulmoogra oil administered by this method have been reported (Dyer,⁸ Hopkins,⁹ Connel,¹⁰ Hollmann and Dean¹¹). The most comprehensive report published on the oral administration of chaulmoogra oil in leprosy is that of Hopkins, who gives the results of its use during fifteen years at the Leper's Home of Louisiana. Excluding cases on which a fair trial of the treatment had not been given, his figures are:

1. Incipient cases	82
Discharged cured	17%
Remaining at the Home, all lesions having disappeared..	4%
Remaining at the Home in an improved condition.....	24%
Absconded in an improved condition.....	24%
Remaining at the Home, the disease apparently arrested..	14%
Worse	8%
Died	4%
2. Advanced cases	88
Remaining at the Home in an improved condition.....	12%
Remaining at the Home, the disease apparently arrested..	5%
Absconded in an improved condition.....	9%
Little change	28%
Disease became terminal.....	20%
Died in the terminal stage.....	23%

Since gastric disturbance and slowness of action are serious obstacles to treatment by oral administration, a number of mixtures of chaulmoogra oil with other substances have been proposed to render it more fluid, improve its absorption and reduce its irritating property, thus making it suitable for subcutaneous and intramuscular injection (Jeanselme,¹² Brocq and Pomaret,¹³ Mercado and Heiser,¹⁴ Hollmann and Currie¹⁵). Of these mixtures, that of Mercado and Heiser,¹⁴ generally known as the Heiser mixture, has been used most extensively. This mixture consists of chaulmoogra oil, 60 c c; camphorated oil, 60 c c, and resorcin, 4 gm.

In 1913 Heiser¹⁴ reported the apparent cure of two cases of leprosy by subcutaneous injections of this chaulmoogra oil mixture, combined with vaccine treatment, and in one case with the oral administration of chaulmoogra oil. Later¹⁵ he reported the apparent cure of two other cases by subcutaneous injections of chaulmoogra oil mixture alone for a period of from 4 to 8 months. In a third communication¹⁶ Heiser gave the results of this method of treatment in 12 additional cases. Some patients were apparently cured, others showed great improvement, and in all the disease had been arrested.

The subcutaneous or intramuscular injection of chaulmoogra oil mixtures has been used subsequently in the treatment of leprosy by Hopkins⁹ in 9 cases.

⁸ N. Y. Med. News, 1905, 87, p. 199.

⁹ New Orleans Med. and Surg. Jour., 1916, 69, p. 223.

¹⁰ Jour. Trop. Med. and Hyg., 1919, 22, p. 37.

¹¹ Jour. Cutan. Dis., 1919, 37, p. 367.

¹² Presse méd., 1911, 19, p. 989.

¹³ Bull. Soc. franç. de dermat. et syph., 1913, 24, p. 70.

¹⁴ U. S. Pub. Health Rept., 1913, 28, p. 1855.

¹⁵ Ibid., 1914, 29, p. 21.

¹⁶ Ibid., p. 2763; Am. Jour. Trop. Dis. and Prev. Med., 1914, 2, p. 300.

by McCoy and Hollmann¹⁷ in 42 cases, by Armellini¹⁸ in 1 case, by Bercovitz¹⁹ in 14 cases, by Coghill²⁰ in 7 cases, by Hall²¹ in 90 cases, by Cadbury²² in 26 cases, by Connel,²³ Hollmann and Dean²⁴ and others, with encouraging results. The last named authors report 12 leper patients in Hawaii who became bacteriologically negative after treatment by intramuscular injections of chaulmoogra oil mixtures, of which only 2 subsequently had a recurrence, one within 7 months and the other within 2 years.

Recently Hollmann and Dean²⁴ have prepared and used by intramuscular injection the ethyl esters of fractions of the fatty acids of chaulmoogra oil in the treatment of leprosy in Hawaii. These acids, after isolation by the ordinary chemical methods, were separated into fractions by fractional crystallization and the fractions converted into their ethyl esters. The four fractions used in the experiments were:

- A. Ethyl ester of chaulmoogric acid.
- B. Ethyl ester of acids crystallizing from alcohol with chaulmoogric acid in the initial separation.
- C. Ethyl ester of acids soluble in 92% alcohol in the first separation and which form soluble lead salts.
- D. Ethyl esters of acids forming lead salts insoluble in ether.

These authors report 26 cases treated 4 months or longer by these several fractions. Patients receiving fractions C and D have shown the greatest improvement. Of these 26 cases, all have shown improvement—many, marked improvement. Eight have already become bacteriologically negative and have been paroled from segregation.

In this connection it is interesting to note that Brill and Williams²⁵ state that the ethyl esters of chaulmoogric and hydnocarpic acids, sometimes known as antileprol, were found to be ineffective on leprosy in the Philippine Islands.

The next advance in the chaulmoogra oil therapy of leprosy was the attempt to devise preparations suitable for intravenous injection. Vahram,²⁴ in order to avoid gastric disturbances resulting from the use of chaulmoogra oil by stomach and the pain and abscess formation incident to subcutaneous injection, prepared an emulsion of chaulmoogra oil for intravenous injection. The formula for this "pseudo-solution," as given by him is: gum acacia, 0.144 gm.; chaulmoogra oil, 0.00072 gm. The oil is added to the gum acacia. After desiccation cold, the mixture is submitted to long porphyzation, then put in suspension in the initial volume of liquid and sterilized at 110 C. The emulsion prepared in this manner is characterized by the minuteness of the suspended globules of oil, which are said to approach the dimensions of colloidal granules. Vahram thinks this property not only renders the emulsion suitable for intravenous injection but that it, in consequence of the great multiplication of surface, should produce an intensification of the therapeutic action. Stévenel,²⁵ in collaboration with Noc, prepared an emulsion for intravenous injection by shaking chaulmoogra oil with N/1 Na₂CO₃. Oil globules of emulsions prepared by this method are said to be as small or smaller than red blood corpuscles.

¹⁷ U. S. Pub. Health Bull., 1916, No. 75, p. 3.

¹⁸ Clin. Dermosifilopat. d. r. Univ. di Roma, 1917, 35, p. 103.

¹⁹ Jour. Am. Med. Assn., 1917, 68, p. 1960.

²⁰ Ann. Trop. Med. and Parasit., 1917, 11, p. 205.

²¹ Trop. Dis. Bull., 1919, 13, p. 13.

²² China Med. Jour., 1918, 32, p. 226.

²³ Philippine Jour. Sc., 1916, Sect. A, 11, p. 78; 1917, Sect. A, 12, p. 207.

²⁴ Progrès med., 1916, 31, p. 19.

²⁵ Bull. Soc. Path. Exot., 1917, 10, p. 684.

Vahram,²⁴ Hopkins⁹ and Stévenel²⁵ each report improvement in two cases of leprosy undergoing intravenous treatment with these emulsions; but apparently these patients had not been under treatment long enough to determine the ultimate result.

Rogers²⁶ appears to be the first to use the soluble salts of the fatty acids of chaulmoogra oil intravenously in leprosy, although such salts and their administration by mouth are mentioned by Roux,¹¹ Trapezinkoff,¹¹ Dyer,⁸ Desprex,²⁷ Amaral and Parambos,²⁸ and Hollmann and Currie.¹¹ Rogers separated the total fatty acids of chaulmoogra oil by the ordinary chemical methods. These crude fatty acids were then separated into fractions of different melting points by dissolving in hot alcohol and removing the acids that crystallized out at different temperatures as the solution cooled. The first fraction, which constituted about two thirds of the total, had a melting point of from 40.8 to 43 C. and is designated as fraction A; the second, with a melting point of from 37 to 40 C., is fraction B, and the remainder, which was liquid at room temperature (28 C.) in Calcutta, is fraction C. These fractions, which may be purified by dissolving in ether, were converted into water-soluble sodium salts by titrating with sodium hydroxid, using phenolphthalein as an indicator, and are called by Rogers "sodium gynocardates."

Rogers states that the sodium salt of fraction A is only slightly soluble in water, is unsuited for either subcutaneous or intravenous injection, and that it is doubtful whether it has any therapeutic value. He used the sodium salts of fractions B and C combined, first subcutaneously and later intravenously²⁹ in the treatment of leprosy. Still later Rogers³⁰ decided that the fraction of the fatty acids of chaulmoogra oil having a higher melting point of from 49 to 62 C. yield sodium salts sufficiently soluble and that they are more potent in the treatment of leprosy than the salts of fractions having lower melting points. After two years' experience with sodium gynocardate in leprosy he found that subcutaneous injections do not produce reactions in leprosy tissues and are less effective therapeutically than intravenous injections. He employed the intravenous route very extensively, having given over 1,000 intravenous injections of the drug, without any ill effects beyond temporary giddiness and headache and occasional localized clotting in the veins, while the results have been most encouraging. All of the patients have shown improvement. The lesions have disappeared and become bacteriologically negative in 50% of the cases treated within 3 years of the onset of the disease, including cases treated for only from 3 to 12 months; while in cases of from 3 to 15 years' duration, 25% have cleared up under treatment.

Rogers' sodium gynocardate has been used subcutaneously and intravenously by Cadbury,³² by Carthew³¹ in 13 cases, by Muir³² in 30 cases, by Peacock³³ in 6 cases, by Rogers in 36 cases, by Connel,¹⁰ Muir,³¹ in part combined with sodium morrhuate, in 23 cases of leprosy with equally encouraging results.

²⁶ Lancet, 1916, 1, p. 288.

²⁷ Lepra, 1900, 6, p. 218.

²⁸ Bull. gén. de therap., 1908, 155, 415; Lepra, 8, p. 249.

²⁹ Brit. Med. Jour., 1916, 2, p. 550.

³⁰ Ind. Jour. Med. Research, 1917, 5, p. 227.

³¹ Ind. Med. Gaz., 1918, 53, p. 407.

³² Ibid., p. 209.

³³ Ibid., p. 95.

³⁴ Ibid., 1919, 54, p. 130.

Spittle³⁵ alone reports unfavorably, stating that the reaction was always more or less severe even after small doses and in no case, even after several months, did benefit result.

Recently Rogers³⁶ reported the results of experiments with the sodium salts of the fatty acids of the oil from the seeds of *Hydnocarpus wightiana*, a plant closely related to *Taraktogenos (Hydnocarpus) kurzii* from which chaulmoogra oil is obtained. This oil is said to contain a larger proportion of hydnocarpic acid than chaulmoogra oil. The results of the treatment with these salts, which he now designates as "sodium hydnocarpate," Rogers considers very satisfactory. There was a great reduction and frequent total disappearance of the lepra bacilli, and the number of cases in which the lesions disappeared in less than a year is considered noteworthy. In only one of fourteen cases was the improvement slight.

The promising results obtained in the treatment of leprosy with chaulmoogra oil and its products would naturally suggest their trial in tuberculosis; yet there have been surprisingly few attempts to apply chaulmoogra oil therapy to this disease. Hernandez³⁷ gives an account of a few experiments, of which only a review has so far been available. He found that the addition of 2% of chaulmoogra oil to the culture medium inhibited the growth of *B. tuberculosis*, and that treatment of experimentally infected guinea-pigs seemed to confirm the destructive action of chaulmoogra oil on tubercle bacilli. Six patients suffering with tuberculosis were treated with small injections (subcutaneous?) of chaulmoogra oil, 1-2 c.c. at from 20 to 30 day intervals; all symptoms are said to have subsided in some of the patients.

Rogers³⁷ suggested the use of sodium gynocardate (chaulmoograte) in the treatment of tuberculosis in 1916; but, since in rare cases of leprosy prolonged febrile reaction and temporary exacerbation of the disease may follow intravenous administration of sodium gynocardate, he hesitated to use it in tuberculosis. For this reason he was led to try sodium morrhuate, the sodium salts of the fatty acids of cod-liver oil, in the treatment of tuberculosis. Without preliminary cultural or animal tests, Rogers has used this salt in the treatment of human tuberculosis, and has supplied it to several clinicians in India for trial, and sodium morrhuate has been put on the market as a specific treatment for tuberculosis.

Rogers¹ states that intravenous injections of sodium morrhuate produce a slight febrile and local congestive reaction, similar to that produced by sodium gynocardate in leprosy, which clearly points to a definite action on the tuberculous tissue. Improvement in phthisical cases is seen in the reduction and cessation of the fever, diminution of the expectoration and cough, and steady gain in weight. In addition, the tubercle bacilli in the sputum gradually decrease in number, and may in time disappear. Moreover, they commonly show deficient acid-fast staining and a granular or beaded appearance, indicating that they are actually being destroyed within the tissues. Furthermore, Rogers says that a year's experience has shown that sodium morrhuate is of great value in leprosy. He believes that the unsaturated fatty acids of both chaulmoogra and cod-liver oils act in some way on the coating of acid-fast bacilli, that of the tubercle bacillus having been shown to contain palmitic and other unsaturated fatty acids.

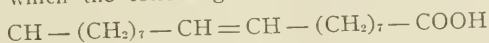
³⁵ *Ibid.*, 1918, 53, p. 33.

³⁶ *Brit. Med. Jour.*, 1919, 1, p. 147.

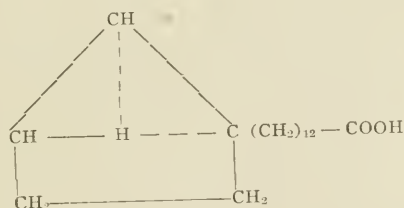
³⁷ *Jour. Am. Med. Assn.*, 1918, 71, p. 1177.

THE CHEMISTRY OF CHAULMOOGRA OIL

Chaulmoogra oil is a fixed oil, expressed cold from the seeds of *Taraktogenos* (*Hydnocarpus*) *kurzii* King, a tree native of Burma. The most complete and trustworthy investigation of the chemistry of chaulmoogra oil is that by Power and collaborators (1904-1907).³⁸ Chaulmoogra oil has a melting point of from 22 to 23 C., a specific gravity of 0.951 at 25 C., an acid value of 23.9, a saponification value of 213.0 and an iodine value of 103.2. It is optically active, (a) $\frac{D}{150} = +52.0$ C. Like other fixed oils and fats, chaulmoogra oil consists essentially of glyceryl triesters of fatty acids. Of chief interest is the fact that Power and his collaborators have isolated from chaulmoogra oil and studied chemically a series of unsaturated fatty acids which have a structure and properties entirely different from all other known fatty acids. Although these fatty acids have the same empiric formula as the fatty acids of the linoleic series ($C_{18}H_{32}O_2$) found in linseed and many other vegetable oils, they differ from these and all other fatty acids in their molecular structure. The fatty acids hitherto known are aliphatic or acyclic compounds, which are optically inactive and have their atoms or radicals arranged in an open chain, of which the following structural formula of oleic acid is an example:



On the other hand, the fatty acids of the chaulmoogric series are optically active and have their atoms arranged in a closed carbon chain or ring; that is, they are cyclic compounds, as is illustrated by the structural formula proposed by Barrowcliff and Power for chaulmoogric acid:



Power and his collaborators isolated two fatty acids of this cyclic series from chaulmoogra oil. One, constituting the larger proportion of the fatty acids, has a melting point of from 68 to 69 C., an empiric formula $C_{18}H_{32}O_2$, and is designated as chaulmoogric acid; a lower isomer, having a melting point of from 59 to 60 C. and an empiric formula of $C_{16}H_{28}O_2$, is designated as hydnocarpic acid. These authors also suggest that chaulmoogra oil may contain other lower isomers of this series of fatty acids. In addition to this series of cyclic fatty acids, chaulmoogra oil contains a small amount of a common, saturated, aliphatic fatty acid, palmitic acid, together with glycerol and phytosterol; and, since the oil is expressed cold from the seeds, it may possibly contain a small amount of the cyanogenic glucosid, gynocardin (Power and Lees) and other nitrogenous substances.

³⁸ Power and Gornall: *Jour. Chem. Soc.*, 1904, 85, p. 851; Power and Lees: *Ibid.*, 1905, 137, p. 349; Power and Barrowcliff: *Ibid.*, 884; Barrowcliff and Power: *Ibid.*, 1907, 91, p. 557; Power, F. B.: *Am. Jour. Pharm.*, 1915, 87, p. 493.

Chattopadhyay³⁰ has taken exceptions to the conclusions of Power as to the cyclic structure of the chaulmoogric acid series; but Brill,²³ investigating the oil of *Hydnocarpus venenata*, a species closely related to *Taraktogenos* (*Hydnocarpus*) *kurzii*, has confirmed the presence of a fatty acid series in this group of plants having a closed carbon chain.

METHODS

The present report is concerned exclusively with a study of the antiseptic and bactericidal actions of chaulmoogra oil and its constituents, the identification and isolation of the bactericidally active substance of chaulmoogra oil, the determination of the specificity of its bactericidal action for acid-fast bacilli, and an investigation of the presence or absence of this bactericidal substance in cod-liver and other oils. The chemotherapeutic investigations on experimentally infected animals will be described separately in the near future.

The methods consist (1) in the separation of chaulmoogra and other oils into fractions and chemical constituents, (2) the preparation of water soluble salts of these fractions and constituents, and (3) tests of the antiseptic and bactericidal activities of these salts against acid-fast and other bacteria.

Since the fixed oils consist essentially of glyceryl triesters of fatty acids, the fundamental analytic procedure has consisted in the separation of the fatty acids from the glycerol and other nonsaponifiable constituents. The ordinary chemical methods of saponifying with alcohol-potash solution and recovering the fatty acids by decomposing the potassium soaps with dilute sulphuric acid were employed. In the case of chaulmoogra oil, the fatty acids, which are solid at room temperature, after being washed free from sulphuric acid, were sometimes purified by dissolving in ether and washing with water and then recovered by evaporating the ether. The fatty acids of cod-liver and linseed oils are fluid at room temperature* and float on the surface of the solution as an oily layer; consequently, a slightly different procedure is necessary to recover the separated fatty acids. This was accomplished by dissolving the fatty acids in ether and washing in a separating funnel.

Rogers' fractions of the fatty acids of chaulmoogra oil, used by him in the treatment of leprosy, were prepared by a modification of the method described by this author. He dissolved the total fatty acids in hot 95% alcohol, and removed those that crystallized out at different temperatures as the solution cooled. The essential point is to obtain successive fractions of the total fatty acids crystallizing out of alcohol and having melting points of from 40.8 to 43 C., 37 to 40 C. and below 28 C., respectively. We have found it more practicable to obtain fractions having melting points within the required limits by fractional crystallization of the total fatty acids from cold solutions in three parts of 95% alcohol. Fraction A, with a melting point of 42 C., is a white crystalline solid; fraction B, with a melting point of 37 C., is a slightly yellowish, somewhat amorphous solid; and fraction C is a light brown fluid at 28 C. and a yellowish pasty mass at from 15 to 20 C.

The isolation and purification of the chemically distinct fatty acids of chaulmoogra oil was done by the methods described by Power and Gornall and Power and Barrowcliff.²⁸ Pure chaulmoogric acid was separated from the total fatty acids by repeated crystallization from 95% alcohol, followed by recrystallization from petroleum ether until a constant melting point of from 68 to 69 C. of the crystals was obtained. This acid, which constitutes the

³⁰ Am. Jour. Pharm., 1915, 87, p. 473.

greater part of the fatty acids of chaulmoogra oil, crystallizes in colorless glistening leaflets, insoluble in water, but sparingly soluble in and readily crystallized from ordinary organic solvents, with the exception of ether and chloroform in which it is readily soluble cold. Pure hydnocarpic acid was obtained from the noncrystalline residue from chaulmoogric acid by fractional precipitation with barium acetate, recovery of the fatty acids by decomposition of the barium salt fractions with dilute hydrochloric acid, and purification of the fractions last precipitated by crystallization from alcohol and finally from petroleum ether until a constant melting point of from 59 to 60 C. was obtained. This acid, like chaulmoogric acid, crystallizes as colorless, glistening leaflets. The palmitic acid fraction, remaining after the removal of the chaulmoogric and hydnocarpic acids, is a light brown oily fluid at room temperature. It undoubtedly contains small amounts of dissolved chaulmoogric and hydnocarpic acids or lower isomers, but it was not found necessary to purify this fatty acid for use in our experiments.

On account of the insolubility of the oils and their fatty acids in water, it was necessary to convert the fatty acids into water soluble sodium or potassium salts, in order to have solutions suitable for bactericidal tests. Rogers^{29, 30} has shown that the therapeutic activity of chaulmoogra oil and its fatty acids is not decreased but rather increased by combining the fatty acids with sodium, due probably to the greater solubility and absorbability of the salts. These soluble salts were prepared by titrating the oil or its fatty acid fractions with half normal sodium or potassium hydroxid, using a suitable indicator. The standard chemical method requires that fatty acids be titrated in hot alcohol with phenolphthalein as an indicator because in aqueous solutions the salts of the fatty acids undergo hydrolytic dissociation. The fatty acid being insoluble is removed from the sphere of chemical action and the sodium ions combining with water interfere with the correct titration. The presence of ethyl alcohol in a concentration of 40% or higher prevents this hydrolytic dissociation and permits a correct titration. In our experience, however, the titration of fatty acids of chaulmoogra oil in 70% alcohol with phenolphthalein as an indicator gives a solution of the sodium or potassium salts which, when diluted with water, is strongly alkaline to litmus, is clear or only slightly clouded, but precipitates on standing and has a low bactericidal activity. On the other hand, when properly titrated in water the solution has a lower titer, is neutral or only feebly alkaline to litmus, is clouded but does not precipitate on standing, and possesses the maximum bactericidal activity. The practical difficulty in titrating in water is that phenolphthalein or any other of a series of indicators tested does not show a sharp end-point, and serves at best only as a rough control of the titration. Consequently, it was necessary to determine experimentally the titer of the chaulmoogrates giving the maximum bactericidal activity. The essential points in the titration are that all of the fatty acids are saponified, that the reaction to litmus is nearly neutral, and that the solution does not precipitate on standing. Since only the sodium and potassium salts of these fatty acids are soluble in water, our tests have necessarily been confined to them.

The correct titer of the oil or its fatty acids having been determined, a 1% solution for testing its antiseptic and bactericidal action was made up as follows: One gram of the oil or fatty acids was accurately weighed and placed in a 100 cc volumetric flask, and the required amount of normal sodium hydroxid and a little distilled water added; the flask and its contents were then heated over a water bath and repeatedly shaken until the fatty acids were

completely saponified. The flask was then filled up to the graduation mark with distilled water and sterilized. This gave a 1% solution, not of the sodium chaulmoograte, but of the oil or fatty acids.

A 1% solution of the oil or fatty acids, instead of a 1% solution of the salts of the fatty acids, was made because we believe that in all comparative bactericidal and chemotherapeutic tests comparison should be made of the bactericidally and chemotherapeutically active atoms or radicals and not of the whole compound containing varying kinds and amounts of bactericidally and chemotherapeutically inert atoms and radicals. The latter serve to render the active atoms or radicals more soluble, absorbable and parasitotrophic, or less irritating and toxic to the host. The sodium in the sodium chaulmoogrates serves only to render the fatty acids soluble and plays no direct part in the bactericidal activity of the compound, as shown by the facts that potassium can replace sodium and that any excess of either base over that necessary to secure solution of the fatty acids, even within the limits of chemical combination with the fatty acids, depresses the bactericidal activity of the solution.

For testing the antiseptic and bactericidal activity of chaulmoogra and other oils and their constituents, cultures of the following acid-fast bacilli were employed: *B. leprae muris* (Hollmann); *B. leprae hominis* (Levy); *B. smegmatis*; *B. lymphangitidis bovis* (Traum); *B. tuberculosis avis*; *B. tuberculosis bovis* and *B. tuberculosis hominis*. Allied to the acid-fast bacilli are the streptothrices, filamentous branching fungi, often having a bacillary stage and a more or less acid-fast staining reaction, and causing streptothriciases in man and animals. A considerable series of these organisms have been used in our experiments, including one or more strains of the following species: *Streptothrix asteroides*, *S. caprae*, *S. eppingeri*, *S. hominis*, *S. madurae* and *S. nocardii*. For determining the specificity of the bactericidal action of the chaulmoogrates against acid-fast bacilli, cultures of the following nonacid-fast bacteria were used: *B. coli*, *B. typhosus*, *B. dysenteriae* Shiga, *B. mucosus*, *B. pestis*, *Spirillum cholerae-asiaticae*, *Staph. aureus* and *Streptococcus* (non-hemolytic). We are indebted to Dr. K. F. Meyer of this laboratory for many of these cultures, and to Dr. J. Traum of the Veterinary Division of the Department of Agriculture, University of California, for a culture of *Bacillus lymphangitidis bovis*, a more or less acid-fast bacillus which he has found in and isolated from a type of chronic lymphangitis in cattle.

Our experiments with the cultures from human and rat leprosy are open to a certain criticism. A considerable series of more or less acid-fast organisms have been cultivated by different investigators from the lesions of human and rat leprosy, none of which have proved to be identical with *B. leprae* Hansen or the variety of it found in rat leprosy. The most that can be said of our cultures is that they are acid-fast bacilli cultivated by competent bacteriologists from the lesions or blood of human or rat leprosy, and, in the case of the culture from rat leprosy, that the organism was found to be pathogenic for rats, in which it produces a disease similar to rat leprosy. However, the criticism to which these cultures are open is largely, if not wholly, met by the fact that the bactericidal activity of the chaulmoogric acid series has been found to be specific for all members of the acid-fast group of bacteria.

The *in vitro* method of testing germicidal action of drugs as a guide to their use in chemotherapeutics has fallen into disfavor, because it has been found that germicidal action in the test tube does not always mean germicidal activity in the animal body in dosage within the limits of tolerance of the patient. However, sweeping criticism of this method is unjustified and is based

on a misconception of its purpose and limitations. The *in vitro* method of testing germicidal action is an analytic method which enables us to exclude the organotrophic reactions of the host and consequently to obtain uncomplicated information on the action of the drug on the parasite. Moreover, and very important for our purpose, the use of this method has enabled us to exclude the possible indirect or physiologic actions of chaulmoogra oil in leprosy and obtain exact data on the bactericidal activity of this oil and its constituents against acid-fast bacteria.

The tests of the antiseptic action of chaulmoogric and other fatty acids were made by adding with a sterile pipet, graduated to hundredths c.c., the required amounts of a sterile 1 or 0.1% solution of the salts to a series of tubes or flasks containing definite quantities of suitable fluid culture medium, so that dilutions of 1:1,000 up to the limits of antiseptic action were obtained. These culture tubes or flasks, together with controls of plain medium, were then inoculated with the organism to be tested, the openings sealed to prevent evaporation and incubated at 37.5 C. for at least twice the length of time necessary to obtain the maximum growth in the controls. These cultures were examined from time to time and the amount of growth or absence of growth recorded and, at the termination of the experiment, microscopic examination for purity was made of the cultures showing growth.

Another method was employed to determine the limits of bactericidal action, since in the antiseptic tests growth might be inhibited without the bacteria being killed. Culture tubes containing measured amounts of suitable culture medium were inoculated with the organism to be tested and incubated until a slight but distinct growth had developed; then the proper dilutions of the fatty acid salts were added to the growing cultures, excepting controls, the tubes replaced in the incubator, and at definite intervals transplants of one small loopful were made from each tube to tubes of fresh mediums, which were incubated and the growth results recorded. The amount of the bactericide transferred by this method to the transplant culture gave a dilution in every case far beyond the limits of antiseptic action. This method, instead of the standard method of testing bactericidal action, was used in our work because growth of the several varieties of tubercle bacilli after submergence and drying is uncertain. In the case of *B. tuberculosis* another method was used in an attempt to control the limits of bactericidal action. To a series of tubes containing 10 c.c. of a suspension of tubercle bacilli in salt solution definite dilutions of the chaulmoogrates were added, two tubes being reserved as controls. After incubation at 37.5 C. for 24 hours, 0.5 c.c. of the suspension of tubercle bacilli from each tube, well shaken up, was injected subcutaneously into guinea-pigs. The bactericidal action of the different dilutions of chaulmoogrates was determined by the absence or presence of infection at necropsy of the guinea-pigs several months after inoculation.

EXPERIMENTAL DATA

1. ANTISEPTIC AND BACTERICIDAL ACTIONS OF THE TOTAL FATTY ACIDS OF CHAULMOOGRA OIL

Rogers,³¹ Hollmann and Dean¹¹ and others claim superior therapeutic results in leprosy from the use of the salts or esters of the fatty acids of chaulmoogra oil; therefore it seemed probable that the active therapeutic principle of this oil must be contained in the fatty acid fraction. Consequently the antiseptic and bactericidal properties of the total fatty acids of chaulmoogra oil were first investigated. In our preliminary experiments the total fatty acids were titrated in water with N/1 Na_2CO_3 , using phenolphthalein as indicator, and

the tests were made on Hollmann's bacillus of rat leprosy cultivated in glycerol veal broth. The results of the first three experiments, in which successively higher dilutions were tested to determine the limits of antiseptic action, and in which transplants were made to determine whether or not the inhibited cultures were actually killed, are combined in table I.

TABLE 1

PRELIMINARY TESTS OF THE ANTISEPTIC AND BACTERICIDAL ACTIONS OF THE SODIUM SALTS OF THE TOTAL FATTY ACIDS OF CHAULMOOGRA OIL ON *B. LEPRÆ MURIS*

Proportion of Chaulmoogric Acids Added to Culture Medium	Growth in Treated Cultures		Growth in Transplant Cultures
	Macroscopic	Microscopic	
From 1:1,000 to 1:60,000	0	0	0
1:70,000	0	0	+
1:80,000	0	0	0
1:90,000	0	0	0
1:100,000	0	0	0
1:125,000	+	+	+
1:150,000	+	+	+
1:175,000	+	+	++
1:200,000	+	+	+++
1:500,000	+	+	++++
1:600,000	++	++	++++
1:700,000	++	++	++++
1:800,000	++	++	++++
1:900,000	++	++	++++
1:1,000,000	+++	+++	++++
1:1,500,000	++++	++++	++++
1:2,000,000	+++++	+++++	+++++
Controls	+++++	+++++	+++++

From these results it appears that the sodium salts of the total fatty acids of chaulmoogra oil have markedly antiseptic and bactericidal actions on Hollmann's culture of rat leprosy bacillus. Growth of the cultures was totally inhibited up to the dilution of 1:125,000 and partial inhibition, as shown by feeble or delayed growth, extended up at least to the dilution of 1:1,000,000. Complete bactericidal action, as shown by absence of growth in transplants to fresh culture medium, extended up to the dilution of 1:100,000, with the exception of 1:70,000 which showed growth. The tendency of chaulmoogrates to skip antiseptic and bactericidal action is characteristic and will be discussed later.

In the next series of antiseptic tests of the sodium salts of the total fatty acids of chaulmoogra oil, the fatty acids were titrated in 70% alcohol with N/2 NaOH using phenolphthalein as indicator, according to the standard chemical method. The titer of 1 gm. of the fatty acids was 3.8 cc N/1 NaOH. The tests were made, as in the first series, against the bacillus of rat leprosy in glycerol veal broth. The antiseptic action of this solution proved by repeated tests to be only about one-half that of the first solution titrated in water. The cause of this depression of the antiseptic action of the fatty acids titrated in alcohol was obscure. It seemed possible that it might be due (1) to a higher initial acidity of the culture medium, perhaps increased by the growth of the cultures, sufficient to precipitate some of the fatty acids; or (2) to the high titer of the chaulmoograte solution titrated in alcohol. Titration of the culture medium before and after the growth of the cultures in it showed that the reaction was the same in both series of tests and that the growth of the rat leprosy bacillus decreased rather than increased the initial acidity of the medium. Com-

parative experiments were then undertaken to determine the influence of the reaction of the culture medium and of the titer of the chaulmoogrates on the antiseptic action of the sodium chaulmoogrates against *B. leprae muris*, the results of which are recorded in table 2.

TABLE 2

THE INFLUENCE OF VARIATIONS IN THE TITER OF THE FATTY ACIDS AND IN THE REACTION OF THE CULTURE MEDIUM ON THE ANTISEPTIC ACTION OF THE CHAULMOOGRIC ACIDS ON *B. LEPRAE MURIS*

Experiment	Titer of the Fatty Acids N/1 NaOH	Reaction of the Culture Medium	Dilution of the Chaulmoogric Acids Totally Inhibiting Growth*
5	1.1	+1.65	—1:20,000
6	1.8	+3.45	1:130,000
7	1.8	+1.7	+1:100,000
8	2.05	+1.65	1:90,000
9	2.05	+1.65	1:130,000
10	2.5	+2.0	1:40,000
11	3.0	+2.0	1:50,000
12	3.6	+1.65	+1:50,000
13	3.8	+1.7	—1:50,000
14	{ 1.8	+1.7	1:100,000
	{ 1.8	0	—1:70,000
	{ 3.8	+3.45	+1:50,000
15	{ 3.8	+1.65	—1:50,000
	{ 3.8	0	+1:25,000
16	{ 4.1	+1.7	1:30,000
	{ 4.1	0	—1:30,000

* In this column the minus sign indicates lower and the plus sign higher than the figures.

From table 2 it is apparent, first, that there is some fluctuation in the antiseptic action with the same titer of the chaulmoogric acids and the same reaction of the culture medium. This fluctuation is due in part to the before-mentioned tendency of the chaulmoogrates to skip antiseptic action and is probably wholly explainable on the same grounds, which will be discussed later. But apart from these fluctuations there are more marked and constant variations in antiseptic action which plainly correspond to the difference in titer of the chaulmoogric acids and in the reaction of the culture medium. These variations would have shown up in greater contrast had it not been that some of the lower, higher and intermediate ranges of antiseptic action were not determined, owing to contaminations of the cultures or to the fact that lower or higher dilutions of the chaulmoogric acids were not included in the tests. It appears that a titer of 1.8-2.05 cc of N/1 NaOH per cc of the total fatty acids of chaulmoogra oil gives the maximum antiseptic activity against *B. leprae muris*, and other experiments have proved that this is true for other acid-fast bacilli. This is the titer obtained by titration in water. Any considerable increase or decrease in the titer from 2.0 markedly depresses the antiseptic and bactericidal activity of the chaulmoogric acids. These experiments also show that a reduction of the acidity of the culture medium to zero likewise depresses the antiseptic activity of the chaulmoogrates. This is brought out specially in Exper. 14, 15, and 16 (table 2), which are strictly comparative tests in the same lot of culture medium with the reaction modified.

This most active titer of 2.0 does not correspond to the titer required to convert all of the chaulmoogric acids into normal sodium salts, as indicated by their titration in alcohol with phenolphthalein as indicator, which requires

a titer of 3.6-3.8. The hydrogen-ion concentration of 1% solutions of the chaulmoogrates at different titers, which were made by the colorimetric method of Clark and Lubs,⁴⁰ show the same divergence between the titer of 2.0 and the neutral point as does the titration method, but throws no light on the cause of the greater antiseptic and bactericidal activity of solutions of the lower titer.

TABLE 3
THE HYDROGEN-ION CONCENTRATION OF 1% SOLUTIONS OF SODIUM CHAULMOOGRAATES AT
DIFFERENT TITERS OF N/1 NaOH

Titer of Solution	Hydrogen-Ion Concentration with Indicators			
	Methyl Red (Range 4.4-6.0)	Brom Cresol Purple (Range 5.2-6.8)	Brom Thymol Blue (Range 6.6-7.6)	Phenol Red (Range 6.8-8.4)
2.0	5.6	5.4
3.0	...	6.4	6.4	...
3.8	7.6	7.7

From the tests in table 3 it appears that the solutions of a titer of 2.0 have a hydrogen-ion concentration well on the acid side, while the titer of 3.8 is slightly on the alkaline side.

A possible explanation of the greater antiseptic and bactericidal activity of the neutral solutions with a titer of 2.0 in water over the alkaline solutions with a titer of 3.8 in alcohol is supplied by Power and Gornall.²⁸ These authors found that in strongly alkaline alcoholic solutions of chaulmoogric acid the normal potassium salt ($C_{18}H_{31}O_2K$) was formed, while in neutral aqueous solutions the acid potassium salt ($C_{18}H_{31}O_2K - 2C_{18}H_{32}O_2$) was formed. Presumably the same reactions would hold true for the sodium salts, in which case the greater bactericidal activity of the neutral, water titrated solution would be due to the acid sodium salts of the chaulmoogric acids.

Having determined the titer of the total fatty acids of chaulmoogra oil that gave the highest antiseptic activity against *B. leprae muris*, we proceeded to test its antiseptic action on other acid-fast bacilli in cultures. In the case of the several varieties of *B. tuberculosis* certain technical difficulties were encountered. While our cultures of other acid-fast bacilli will grow in glycerol veal broth in intimate contact with the antiseptic solution, the human and bovine varieties of *B. tuberculosis* must be inoculated and grow only on the surface of the culture fluid as a waxy membrane. Owing to this growth requirement of tubercle bacilli and to the fatty nature of the bacilli, only the lower surface of the inoculated fragment of membrane comes in contact with and is made wet by the culture fluid and contained antiseptic. The chaulmoogrates are not freely soluble and gradually crystallize out of solution; it is remarkable that this tendency to crystallize out appears to be more marked in weaker solutions. Consequently, in such slow growing cultures as the tubercle bacillus, precipitation of the feebly soluble chaulmoogrates, and possibly also fixation by the contiguous layers of tubercle bacilli, will sufficiently reduce the concentration of the chaulmoogrates in the culture medium to enable the upper layers of tubercle bacilli in the inoculated fragment of membrane that have not been in contact with the antiseptic, to multiply. We have been able to overcome this difficulty in the case of the avian variety of tubercle bacilli which, unlike the human and bovine varieties, can be induced to grow at the bottom of the flask in intimate contact with the antiseptic solution.

⁴⁰ Jour. Bacteriol., 1917, 2, pp. 1-34.

In table 4 are collected the results of antiseptic tests of the sodium salts of the total fatty acids of chaulmoogra oil on different acid-fast bacilli, including comparative tests in surface and submerged cultures of the bacillus of avian tuberculosis. In order that the degree of antiseptic activity of the chaulmoogrates may be appreciated, we have included in another column of this table the antiseptic action of phenol tested under the same conditions on certain of these acid-fast bacilli. The figures are based in every case on the results of repeated tests.

TABLE 4
THE ANTISEPTIC ACTION OF SODIUM CHAULMOOGRATES AND OF PHENOL ON CERTAIN
ACID-FAST BACILLI

Bacilli	Limits of Complete Antiseptic Action		
	Sodium Chaulmoogrates		Phenol
	In Cultures on the Surface of the Broth	In Cultures Submerged in the Broth	In Cultures Submerged in the Broth
<i>B. leprae muris</i> (Hollmann)....	1:80,000 to 1:130,000	1:1,000
<i>B. leprae hominis</i> (Levy).....	1:60,000 to 1:130,000	
<i>B. smegmatis</i>	1:80,000 to 1:110,000	1:1,000
<i>B. lymphangitidis bovis</i>	1:90,000 to 1:130,000	
<i>B. tuberculosis avis</i>	1:10,000	1:90,000 to +1:140,000	1:1,000
<i>B. tuberculosis bovis</i>	1:10,000 to 1:20,000*	
<i>B. tuberculosis hominis</i>	1:10,000 to 1:20,000*	

* Will not grow submerged in the broth.

These results show that the sodium salts of the total chaulmoogric acid have a high antiseptic action on all of the acid-fast bacilli tested, but that this antiseptic attains its full activity only when the organisms are growing in complete contact with it in submerged cultures. Comparison of the antiseptic action of the chaulmoogrates on the surface cultures of the human and bovine varieties with that on the surface cultures of the avian variety of *B. tuberculosis*, and of the surface cultures with the submerged cultures of the avian variety, apparently justifies the conclusion that the chaulmoogrates would have an equally high antiseptic action against the human and bovine varieties if they could be cultivated submerged in and in intimate contact with the antiseptic. Comparison of the chaulmoogrates with the standard antiseptic and bactericide, phenol, brings out in a striking manner the remarkably high antiseptic activity of the former against acid-fast bacilli.

Our experiments with the potassium salts of the total fatty acids of chaulmoogra oil have shown that their antiseptic activity is no greater than, and probably slightly inferior to, the sodium salts. The other ordinary salts of the chaulmoogric acids are insoluble in water and are consequently unsuited for experiments in vitro.

Having established the high antiseptic activity of the sodium chaulmoogrates against acid-fast bacilli, we attempted to determine whether this antiseptic action was merely an inhibition of growth or whether the bacilli were actually killed. We already had, it is true, some evidence that the chaulmoogrates are actually bactericidal and not merely inhibitory of growth. In our preliminary experiments in table 1 transplants from cultures containing chaulmoogrates up to a dilution of 1:100,000 (with the exception of 1:70,000) failed to grow. However, more accurate data on this subject can be obtained

by adding the dilutions of chaulmoogrates to the tubes or flasks of culture medium after inoculation and incubation of the cultures until a definite growth has developed, and then transplanting from the treated cultures to fresh medium at definite intervals. The results on the bactericidal action of the sodium chaulmoogrates on *B. leprae muris* are given in table 5.

In this experiment the sodium chaulmoogrates killed the bacillus of rat leprosy in 24 hours up to a dilution of 1:75,000 but not at a dilution of 1:100,000 of the chaulmoogric acids. These results do not vary greatly from those obtained in connection with the antiseptic tests recorded in table 1, which gave a bactericidal action up to the dilution of 1:100,000. Probably the limits of complete bactericidal action in vitro of the chaulmoogric acids on this bacillus lie somewhere between 1:75,000 and 1:100,000. The experiment in table 5 also shows that action of the chaulmoogrates for a period of time longer than 24 hours does not increase its bactericidal range. No attempt was made to determine the bactericidal activity of the chaulmoogrates acting for a shorter time than 24 hours because there are reasons for believing that the bactericidal action of the chaulmoogric acids is biologic rather than directly chemical and is consequently slow in action.

An attempt was made to obtain data on the bactericidal action of the chaulmoogrates on *B. tuberculosis hominis* by the combined in vitro in vivo method. This consisted in subcutaneous inoculations into a series of guinea-pigs of 0.5 c.c. of saline suspensions of the tubercle bacilli which had been subjected to the action of definite dilutions of the chaulmoogrates for 24 hours at 37.5 C., together with controls inoculated with the same amount of untreated saline suspensions of the bacillus. The results of this experiment as determined by necropsies of the animals are given in table 6.

Some of the guinea-pigs in table 6 died from unknown cause too soon after inoculation to show tuberculous lesions, but these deaths occurred by chance at points in the series at which they do not interfere with the interpretation of the results. This experiment shows a complete bactericidal action of the sodium salts of the total fatty acids of chaulmoogra oil on *B. tuberculosis hominis* under the conditions of the experiments, only up to a dilution of 1:20,000. There is, however, a probable source of error in the conditions of the experiment that should be pointed out. There are reasons, which will be discussed more in detail later, for believing that the bactericidal action of the chaulmoogrates on acid-fast bacilli is not directly chemical by corrosive or fixative action on their protoplasm, as are salts of the heavy metals; but that its action is indirect or biologic, and is dependent on vital activities of the multiplying bacilli, which attach the chaulmoogric acids to themselves for the purpose of synthesizing their fatty envelopes, and that only when this assimilation has occurred can these peculiar fatty acids exercise the toxic action on the bacilli. Tubercle bacilli suspended in salt solution have their vital activities reduced to a minimum, and would consequently be capable of fixing but little of the chaulmoogrates; the bactericidal action would therefore be greatly reduced.

2. THE ACTIVE BACTERICIDAL PRINCIPLE OF CHAULMOOGRA OIL

Crude chaulmoogra oil has usually been recommended as more effective than the refined product in the treatment of leprosy. If this be true, and since the oil is expressed from the seeds, it might be that small amounts of nitrogenous or other nonfatty substances, such as the glucosid, gynocardin, would be expressed with the oil, and constitute the bactericidal and therapeutic active principle of chaulmoogra oil. Brill and Williams²³ found, on analysis of

eight samples of chaulmoogra oil, a small amount of nitrogen present which would correspond to from 0.113 to 0.568% of gynocardin. However, our anti-septic and bactericidal tests and the therapeutic experience of Rogers and others indicate that the active principle resides in the fatty acid fraction of the oil; for the method of separation of the fatty acids and their purification by ether would exclude all but the smallest traces of other substances. In order to confirm this and to identify, if possible, the particular fatty acids that are active, the total fatty acids, the several fractions used by Rogers in the treatment of leprosy, and the individual fatty acids were separated by appropriate chemical methods; the whole oil was saponified, and the several fractions and pure fatty acids were converted into sodium salts and their anti-septic activity tested on acid-fast bacilli. The results of these comparative tests on *B. leprae muris* are given in table 7.

The data in table 7 do not give us the clean cut identification of the active substance of chaulmoogra oil expected from these comparative tests. From the uninterpreted results of these tests it would appear that the total fatty acids are more active than any of its fractions or constituent fatty acids. These apparently paradoxical results are due to certain physical properties of the fractions of higher melting points and of the pure fatty acids which modify the antiseptic and bactericidal activity of these fractions in solutions. Four factors must be considered for the proper interpretation of these data: (1) a bactericidally active fraction; (2) presumably one or more inactive fractions that dilute the active fraction; (3) the low solubility of the salts of fractions having high melting points (Rogers' fractions A and B) and of pure chaulmoogric and hydnocarpic acids, and their consequent tendency to precipitate out of solution; and (4) the well-known physical facts that in mixtures of fatty acids of different melting points, the melting points of the constituent fatty acids are lowered and the solubility of their salts increased.

With these complicating factors in mind the interpretation of the data in table 7 is less difficult. First, it is evident that the active bactericidal principle of chaulmoogra oil is contained in the fatty acid fraction because of the greatly superior activity of the total fatty acids over the whole oil. Second, the progressive increase in activity of Rogers' fractions A, B and C is due to a corresponding progressive decrease in the melting point and increase in solubility of their salts. The increase in the activity of fractions A to C is probably less than it would be if the activity of fractions B and C were not in part neutralized by the increasing proportion of the inactive fraction. Third, fractions B + C are slightly more active than either fraction B or C separately, because the mixture depresses the melting point and increases the solubility of the salts of fraction B which contains a larger proportion of the active fatty acids. Fourth, the last three fractions in table 7 contain together the total fatty acids of chaulmoogra oil which, as we have seen, must contain the active bactericidal substance; yet the activity of each of the three fractions by itself is greatly inferior to that of the total fatty acids. The residue consists chiefly of palmitic acid with some chaulmoogric and hydnocarpic acids or lower isomers that could not be crystallized out of solution. This palmitic acid or other unidentified constituent of the residue cannot be the active substance for the mixture is fluid and its salts freely soluble and, moreover, about 90% of the chaulmoogric and hydnocarpic acids, assumed for the purpose of our argument to be inactive, have been removed, therefore the remaining 10% of theoretical active substance, although not strictly pure, should have an anti-septic activity much greater than that of the total fatty acids. On the contrary,

our tests show that its activity is feeble. The slight antiseptic activity the residue possesses is undoubtedly due to the small amounts of chaulmoogric and hydnocarpic acids contained in it. By this process of elimination we are forced to conclude that the bactericidal activity of chaulmoogra oil is a function of the chaulmoogric acid series, chaulmoogric acid and its isomer, hydnocarpic acid, which are of unique chemical structure among fatty acids, and which constitute about 90% of the total fatty acids of chaulmoogra oil. The feeble antiseptic and bactericidal activity displayed by salts of the pure acids is due to the relatively high melting points and low solubility of their salts, which tend to precipitate out of weak solutions before their bactericidal action becomes effective. When they, together with the small palmitic fraction, are mixed, the melting point of the mixture is depressed and their salts rendered sufficiently soluble to permit the chaulmoogric acid series to exert the high antiseptic and bactericidal activity characteristic of the total fatty acids of chaulmoogra oil.

3. SPECIFICITY OF THE BACTERICIDAL ACTION OF THE CHAULMOOGRIC ACIDS AGAINST ACID-FAST ORGANISMS

Our experiments have shown that the cyclic fatty acids of chaulmoogra oil have a high antiseptic and bactericidal activity against acid-fast bacilli. Is this action specific for acid-fast organisms, or is it general against all bacteria? In order to determine this important point experiments have been conducted with two groups of organisms, the streptothrices and nonacid-fast bacteria. The streptothrices are a group of branching, filamentous fungi, which may by fragmentation develop bacillary forms, and some of them are more or less acid fast. These organisms are regarded by recent investigators as phylogenetically related to the acid-fast bacilli which also, under certain conditions, develop branching forms. Because of this supposed relationship and the variable acid resistance of the different species, the antiseptic and bactericidal action of the chaulmoogrates on this group of organisms is of interest. Table 8 gives the antiseptic action of the sodium salts of the total fatty acids of chaulmoogra oil on different species of streptothrix, together with their morphologic characters and acid-resisting property.

It appears from the results in table 8 that the chaulmoogric acid series have some antiseptic action against streptothrices which is, however, less than against the acid-fast bacilli; that this antiseptic action varies for different species; and that in general the antiseptic action is greater against the bacillary and more or less acid-fast species than against the filamentous and nonacid-fast species.

More interesting and important are the tests of the antiseptic action of the chaulmoogric series on nonacid-fast bacteria (table 9).

These experiments show that the sodium chaulmoogrates are antiseptically and consequently bactericidally inert against nonacid-fast bacteria in dilutions as low as 1:1,000. At such a dilution the growth is usually as luxuriant as in the controls. Dilutions lower than 1:1,000 were not tested, since at this dilution the contrast between the inactivity against nonacid-fast bacteria and the activity against acid-fast bacteria is sufficiently well marked to prove the specificity of the bactericidal activity of the chaulmoogric acids for the latter group of bacteria.

Rogers^{1, 36} has stated that therapeutic activity in leprosy and tuberculosis is not peculiar to the fatty acids of chaulmoogra oil, but is common to the unsaturated fatty acids of cod-liver, and presumably other oils. He suggests that the unsaturated fatty acids act on acid-fast bacilli, the coating of which

TABLE 8
THE ANTISEPTIC ACTION OF SODIUM CHAULMOOGRATES ON STREPTOTHRIX

Streptothrix	Morphology	Acid Resistance	Growth in Chaulmoogrates at Dilutions of										
			1:5,000	1:10,000	1:20,000	1:30,000	1:40,000	1:50,000	1:60,000	1:70,000	1:80,000	1:100,000	Control
S. eppingeri.....	Rods and short filaments	Partial	0	0	0	0	0	0	0	0	+	+	+
S. caprae.....	Rods and short filaments	Partial	0	0	0	0	+	+	+	+	+	+	+
S. noreardi.....	Rods and short filaments	Partial	0	0	0	+	+	+	+	+	+	+	+
S. hominis.....	Long branching filaments	Vegetative	0	0	+	+	+	+	+	+	+	+	+
S. asteroides.....	Long branching filaments	Vegetative	0	0	+	+	+	+	+	+	+	+	+
S. albus.....	Long branching filaments	Vegetative	0	0	+	+	+	+	+	+	+	+	+
S. bovis.....	Long branching filaments	Vegetative	0	+	+	+	+	+	+	+	+	+	+
S. madurae.....	Long branching filaments	Vegetative	0	+	+	+	+	+	+	+	+	+	+

TABLE 9
THE ANTISEPTIC ACTION OF SODIUM CHAULMOOGRATES ON NONACID-FAST BACTERIA

Bacterium	Growth in Sodium Chaulmoogrates: Dilutions from 1:1,000 to 1:100,000	Control
B. coli.....	+	+
B. typhosus.....	+	+
B. dysenteriae (Shiga).....	+	+
B. mucosus.....	+	+
B. pestis.....	+	+
S. cholerae.....	+	+
Staphy. aureus.....	+	+
Strep. sp. (non-haemolytic).....	+	+

TABLE 10
COMPARISON OF THE ANTISEPTIC ACTIVITIES OF SODIUM CHAULMOOGRATES, SODIUM LINOLEATES
AND SODIUM MORRHUATES ON ACID-FAST BACILLI

Bacilli	Dilutions Having Complete Antiseptic Action		
	Sodium Chaulmoogrates	Sodium Linoleates	Sodium Morrhuates
B. leprae muris.....	1:80,000 to 1:130,000	—1:1,000 to 1:3,000	1:5,000 to 1:8,000
B. leprae hominis.....	1:60,000 to 1:120,000	1:1,000	1:7,000 to 1:9,000
B. sinogiantis.....	1:80,000 to 1:110,000	1:1,000 to 1:4,000	1:8,000
B. lymphangitidis bovis.....	1:90,000 to 1:130,000	1:4,000	1:3,000
B. tuberculosis avis.....	1:90,000 to +1:140,000	—1:5,000 to 1:9,000	1:3,000 to 1:9,000

has been shown to contain unsaturated fatty acids. It is consequently of interest to compare the antiseptic and bactericidal activity of the fatty acids of cod-liver and other oils with those of chaulmoogra oil.

Linoleic acid, the principal fatty acid of linseed and certain other vegetable oils, has the same empiric formula ($C_{18}H_{32}O_2$) as has chaulmoogric acid, and differs from it only in the arrangement of the atoms in its molecule; the molecule of chaulmoogric acid has a carbon ring structure, while the molecule of linoleic acid has its atoms arranged in an open chain. Since the two fatty acids are the chief constituents of the respective oils, and since the salts of the total fatty acids of chaulmoogra oil have been proved to be more highly antiseptic and bactericidal *in vitro* against acid-fast bacilli than any of its fractions, it has been considered fair to use the sodium salts of the total fatty acids of both oils for comparative tests.

Rogers' sodium morrhuate consists of the sodium salts of the total fatty acids of cod-liver oil. Our knowledge of the chemistry of cod-liver oil is far from exact; but its composition, as is to be expected of an animal oil extracted from an organ having such metabolic activities as the liver, is very complex. A considerable number of fatty acids, including oleic, palmitic, steric, myristic, palmitoleic, gadoleic, erucic and therapeutic acids, together with two alkaloids, asselin and morrhuin, traces of iodine and sometimes bromine, and butylamine, amylamine, hexylamine and hydrodimethyl-pyridine, have been reported as occurring in cod-liver oil. However, all but traces of substances other than the fatty acids would be excluded by the method of preparation of Rogers' sodium morrhuate. So far as is known, cod-liver oil does not contain fatty acids of the chaulmoogric series nor any fatty acids having a cyclic structure.

In table 10 are collected the results of antiseptic tests of sodium linoleates and sodium morrhuates compared with sodium chaulmoogrates on various acid-fast bacilli. In this table, as in the preceding tables, where two figures are given they represent the lowest and highest range of antiseptic action obtained in repeated experiments.

These experiments show that the sodium linoleates and morrhuates have a slight antiseptic action on acid-fast bacilli. This is probably a nonspecific soap action—for the sodium salts of the fatty acids are soaps, of which dilutions up to 1:5,000 give decidedly soapy and up to 1:10,000 perceptibly soapy solutions—in which the fatty capsules of acid-fast bacilli are injured (emulsified) by the more concentrated solutions of the soaps. In consequence of this low antiseptic activity, and since bactericidal action is never greater, and is usually less, than antiseptic action, it has not been considered necessary to test the bactericidal actions of the linoleates and morrhuates on acid-fast bacilli. In strong contrast to this relatively feeble soap action of the linoleates and morrhuates stands the high antiseptic and bactericidal activities of the chaulmoogrates against acid-fast bacilli, activities which these comparative experiments indicate are specific to the cyclic fatty acids of the chaulmoogric series.

DISCUSSION

It is convenient in discussing the experimental data to follow the outline given in the introductory paragraphs, and to determine how much information we have obtained by these experiments *in vitro* bearing on the several problems involved in the fatty acid therapy of leprosy and tuberculosis.

The first problem was the method of therapeutic action of chaulmoogra oil in leprosy. This problem presented three chief possibilities: (1) that the reputed therapeutic effect is due to direct bactericidal action of chaulmoogra oil or some of its constituents on *B. leprae*; (2) that chaulmoogra oil acts indirectly by stimulating the tissues to react against the invading organisms, and (3) that chaulmoogra oil is inactive and improvement of patients following its use is spontaneous. The first of these possibilities, that of direct bactericidal action, was the simplest and most attractive one, and would place chaulmoogra oil or its active constituent among the true chemotherapeutic agents. For this reason the antiseptic and bactericidal activities of chaulmoogra oil and its constituents were first investigated. Our experiments have shown that the sodium salts of the total fatty acids of chaulmoogra oil have a very high antiseptic and bactericidal activity against acidfast bacilli. This bactericidal action extends to a dilution of about 1:100,000, and the antiseptic action is perceptible to a dilution of at least 1:1,000,000. In the light of these results and of the facts that these high antiseptic and bactericidal activities have proved to be peculiar to certain fatty acids of chaulmoogra oil and specific against acid-fast bacilli, as will be discussed, we have not considered it necessary or profitable to investigate hypothetical indirect action of chaulmoogra oil in leprosy; and we believe that it can be concluded with reasonable certainty that any therapeutic action which chaulmoogra oil may have in leprosy is due to its direct antiseptic and bactericidal action on *B. leprae*.

In this connection the question of the relation of bactericidal dilution in vitro to the therapeutic dosage in vivo of the chaulmoogrates naturally arises. If, as is true, the limits of complete bactericidal action in vitro is the dilution of about 1:100,000, but the therapeutic dosage intravenous in leprosy is in the proportion of from 1:2,000,000 to 1:500,000 of the body weight, how can we account for the therapeutic effect claimed in leprosy on the basis of even the high bactericidal action of the chaulmoogrates? Our experiments in vitro have, for the purpose of analysis, intentionally excluded the factors that may be supplied by the host in the action of the chaulmoogrates on acid-fast bacilli in vivo. There are three such factors which, individually or conjointly, might account for the apparent discrepancy between the bactericidally active dilution in vitro and the therapeutically active dosage in vivo. First, other chemotherapeutic agents are known, such as arsphenamin, which act therapeutically in the animal

body at higher dilutions than they do germicidally in vitro. This intensified action in vivo is probably due to some chemical modification of the substance brought about by the tissues of the host. Second, fats and fatty acids are not, like some drugs, rapidly broken down and excreted by the animal body, but are stored in the tissues for metabolic use. Consequently, in the regular and long continued administration of the chaulmoogric acids in the treatment of leprosy there would be an accumulation of the chaulmoogric acids or their esters in the body that might well reach the concentration of bactericidal action. This might explain in part the slow action of the chaulmoogrates in leprosy. Third, the high antiseptic range of the chaulmoogrates, which is perceptible up to a dilution of at least 1:1,000,000, above the complete bactericidal activity may be an important factor in the therapeutic results. Such a high antiseptic action, although incomplete, might in conjunction with the tissue reactions be sufficient to restrain the multiplication of the bacilli; or the inhibitory action of the chaulmoogrates might so injure or reduce the vitality of the parasites that the natural resistance of the host would be able to overcome them. The experimental determination of the actual relation between bactericidal dilution in vitro and therapeutic dosage in animals will be considered in a later article.

The second problem was that of the active principle of chaulmoogra oil. Our experiments have shown conclusively that the bactericidally active principle is contained in the fatty acids of this oil. This is in accord with the experience of Rogers,^{29, 30} Hollmann and Dean¹¹ and others that the fatty acid fraction of chaulmoogra oil is most active therapeutically in leprosy. But, whereas Rogers claims superior therapeutic results with first one fraction and then another of the chaulmoogric acids, we have obtained the highest bactericidal activity in vitro with the salts of the total fatty acids. Our results are due to the greater solubility of the salts of the mixed fatty acids, and do not indicate that all of the fatty acids of chaulmoogra oil are bactericidally active. The low solubility of the salts of the individual fatty acids and their consequent tendency to crystallize out of weak solutions have interfered with the direct identification of the specific fatty acids of chaulmoogra oil possessing this bactericidal property; but by indirect methods of exclusion we have been able to satisfy ourselves that the small palmitic acid fraction is inactive, and that the bactericidal activity is a function of the chaulmoogric acid series, chaulmoogric and hydnocarpic acids and possibly lower isomers of this

series, which together constitute about 90% of the fatty acids of chaulmoogra oil. The chaulmoogric acid series, it will be recalled, have been shown by the researches of Power and Gornall,³⁸ and of Brill²³ to have a peculiar molecular structure containing a closed carbon chain, which is found in no other known fatty acids.

The third problem was the degree of specificity of the bactericidal activity of the chaulmoogric acid series. Bactericides may be either nonspecific, such as phenol and salts of the heavy metals, which act against all bacteria and show no marked variation in their action on different species, except in so far as it may be modified by the development of resistant spores by certain species; or they may be more or less specific and act more strongly against certain species or groups of bacteria, as is the case with ethylhydrocuprein against the pneumococcus group. Our experiments have shown that the chaulmoogric acids belong to the latter class of bactericides, and that they have a very sharply limited group specificity. They possess a high bactericidal activity against all members of the acid-fast group of bacilli and are inactive against all other bacteria tested. This group specificity is probably connected, not with acid-fastness as such nor with any protoplasmic relationship of the different acid-fast organisms, but with the fat metabolism of acid-fast bacilli and the mechanism of the bactericidal action of the chaulmoogric acids. It is known that growths of acid-fast bacilli contain large amounts of fats and waxes (20-37% in case of the tubercle bacilli), which are intimately connected with the bacterial cell in that they are a product of its metabolism, and constitute its protective capsule. Kendall, Walker and Day⁴¹ have shown that acid-fast bacilli produce a soluble lipase in their growth, which is probably concerned in the metabolism of this fatty capsule. Rogers has suggested that the unsaturated fatty acids act on acid-fast bacilli by injuring the protective fatty capsules of acid-fast bacilli. This assumption, he believes, is supported by his observations that the bacilli excreted by patients undergoing treatment show irregular acid-fast staining. Such bacilli, deprived of their protective fatty capsules, would be exposed to the destructive action of the tissues and body fluids. Our experiments *in vitro*, however, have failed to show the slightest action of the sodium chaulmoogrates, in any concentration and acting for any length of time, on the staining characters or morphology of acid-fast bacilli.

⁴¹ Jour. Infect. Dis., 1914, 15, p. 443.

An hypothesis which seems best to explain the mechanism of the bactericidal action of the chaulmoogric acids and their specificity for acid-fast bacilli is that these fat elaborating bacilli attempt to utilize the chaulmoogric acids to build up their fatty capsules, and that these cyclic fatty acids contain a group or an arrangement of atoms which is toxic for the bacterial cell. In the terminology of Ehrlich's side chain theory, we may express this reaction by saying that chaulmoogric acid possesses an haptophore group which becomes attached to the receptor or side chain of the acid-fast bacillus, and a toxophore group which, after attachment, exerts a toxic action on the bacillus. On the basis of this hypothesis, the chaulmoogric acids are not bactericidal against nonacid-fast bacteria because these organisms, not elaborating a fatty capsule, do not necessarily use fats in their metabolism, and consequently do not possess the proper receptors for the haptophore group of the chaulmoogric acids.

This hypothesis would also help to explain the irregularities encountered in our bactericidal experiments *in vitro*, such as skipping and variation in antiseptic and bactericidal action in different tests. It is well known that fat metabolism, as indicated by acid-fastness, is not a constant or even a vitally necessary function of acid-fast organisms, since there are usually a variable number of nonacid-fast individuals in young cultures of acid-fast bacilli, and it has been shown experimentally that acid-fastness can be modified and even wholly suppressed in some species by conditions of growth. In our experimental cultures such nonacid-fast individual bacilli, in which fat metabolism was temporarily dormant, would by our hypothesis be immune to the action of the chaulmoogrates. After the greater part of the chaulmoogrates in the culture had become fixed by the acid-fast organisms or precipitated in virtue of their slight solubility, these nonacid-fast individuals might be able to multiply and resume their fat metabolism and acid-fast property unrestrained.

The fourth and last major problem investigated was that of the presence of bactericidally active fatty acids in other oils. It will be recalled that Rogers has stated that the salts of the unsaturated fatty acids of both chaulmoogra and cod-liver oils, and by implication the unsaturated fatty acids of any oil, are equally efficacious therapeutically in either leprosy or tuberculosis. Our experiments do not support the claim of Rogers. They show that the high specific bactericidal activity against acid-fast bacilli is not a property common to unsaturated fatty acids, but that it is restricted to the cyclic fatty acids

of the chaulmoogric series. The number of fatty acids that we have investigated is limited; but we believe that they have been particularly well chosen. Linoleic acid has an empiric formula ($C_{18}H_{32}O_2$) identical with that of chaulmoogric acid, and differs from the latter only in the arrangement of its atoms in an open chain instead of in a ring. Rogers' sodium morrhuate contains a considerable number of fatty acids, none of which are known to be of cyclic structure, and for which specific therapeutic action in leprosy and tuberculosis is claimed. We have demonstrated that neither of these have any marked bactericidal activity against acid-fast bacilli, but are relatively inert. Therefore we believe the conclusion to be warranted that the specific bactericidal activity of fatty acids against acid-fast bacilli is a function of the carbon ring structure of the molecules of the chaulmoogric acid series, a structure known to exist only in chaulmoogra oil and in oils of certain plants closely related to *Taraktogenous kurzii* from whence chaulmoogra oil is obtained.

These experiments in vitro supply certain definite information on the major problems of the purposed investigation of the fatty acid therapy of leprosy and tuberculosis, namely: (1) the method of therapeutic action of chaulmoogra oil in leprosy; (2) the active principle of chaulmoogra oil; (3) the specificity of its action on the acid-fast group of bacteria, and (4) the limitation of the active principle to the cyclic fatty acids of chaulmoogra oil. Much of this information could not have been obtained by other methods of experimentation; but the actual chemotherapeutic value of the chaulmoogric acid compounds in the treatment of infections due to the acid-fast group of bacilli, especially tuberculosis, remains to be proved by experiments on animals and by clinical experience. It is strongly recommended, however, that clinical trial of the chaulmoogrates in tuberculosis await the results of the animal experiments now in progress; for the indiscriminate use of this drug may arouse false hopes and be not without danger to the patients.

SUMMARY

Chaulmoogra oil contains bactericidal substances that are about one hundred times more active than phenol.

The bactericidally active substances of chaulmoogra oil are the fatty acids of the chaulmoogric series, chaulmoogric and hydnocarpic acids, and possibly lower isomers of this series.

The bactericidal activity of the chaulmoogric acid series is specific for the acid-fast group of bacteria, and inactive against all other bacteria tested.

This specific bactericidal activity against acid-fast bacteria is a function of the carbon ring structure of the molecule of the chaulmoogric acid series which, so far as known, is found only in chaulmoogra oil and in oils of certain plants closely related to *Taraktogenous kurzii*.

The fatty acids of cod-liver oil, the salts of which constitute Rogers' sodium morrhuate, used in the specific treatment of tuberculosis, do not possess the specific bactericidal activity of the chaulmoogric acid series.

These facts supply a scientific basis for the use of chaulmoogra oil and its products in leprosy.

Our experiments do not support the claims of Rogers for sodium morrhuate in the specific therapy of tuberculosis.

The bactericidal activity of the chaulmoogric acids against all members of the acid-fast group of bacilli, together with the clinical results obtained from their use in leprosy, furnish theoretical grounds for the application of the chaulmoogrates to the therapy of tuberculosis.

Experiments on animals are now in progress to determine whether or not the chaulmoogric acid series have any practical value in the chemotherapy of tuberculosis.

A COMPARISON OF THE MORPHOLOGIC, CULTURAL
AND BIOCHEMICAL CHARACTERISTICS OF
B. ABORTUS AND B. MELITENSIS *

STUDIES ON THE GENUS BRUCELLA NOV. GEN. I

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The problem dealing with the possible pathogenicity of *B. abortus* (Bang) to human subjects investigated in the last ten years from various points of view was placed in a new light when, in 1918, Alice E. Evans¹ demonstrated by morphologic, biochemical and serologic studies a close relationship between the organism responsible for infectious abortion of domesticated animals and the so-called *Micrococcus melitensis*, the cause of the well-known undulant or Malta, or Mediterranean fever in man. Moreover, the peculiar latency in tissues and the apparent ubero- and sexotropic character of the two organisms in cattle and goats, respectively, lend additional support to the above contention. To the bacteriologist, however, who obtains his information mainly from the meager descriptions and accounts given in the usual textbooks instead of from a comparative study of authentic cultures in vitro and in vivo, this correlation of facts appears impossible. We mention in this connection the conservative attitude of a number of English bacteriologists, who place the causative organism of Malta fever with the coccus group and fail to recognize the repeated observation that this organism may appear in smears made from young cultures and even from tissue material as a typical short rod. On the other hand, the small microbes found in some forms of infectious abortion have, since the classic studies of Bang and Stribolt,² been accepted as distinct rods which, however, may occasionally appear in exudates as a "coccobacillus." Furthermore, an analysis of the descriptions dealing with the cultural and biochemical characteristics of the two organisms under consideration reveals only differences of minor importance and adds considerable evidence to the conception of a close relationship of *B.*

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* The letter *B.* is used in this series of papers for the suggested genus *Brucella* Nov. gen. and enhances the suggestions made by A. E. Evans (Footnote 1).

¹ Jour. Infect. Dis., 1918, 22, p. 580.

² Ztschr. f. Thier. med., 1897, 1, p. 241.

abortus and "*Micrococcus*" *melitensis*. Irrespective of the fact, that Alice E. Evans supported her conclusions by the presentation of observations on several strains of the two types of bacteria, a number of bacteriologists have expressed to us their inability to accept this new conception. This in part may be due to the unfortunate mistake which Miss Evans committed in correlating *B. bronchisepticus* with *B. abortus* and with "*Micrococcus*" *melitensis*. As is well known, *B. bronchisepticus* is a motile, strongly alkali-producing rod, which is either related to the *pyocyaneus* group (Smith),³ or to *B. pertussis* (Ferry and Noble).⁴

In order to verify the various statements and preparatory to a number of experiments to be considered in this series of papers, we studied the morphology and biochemical reactions of 21 cultures, which had been identified by various authoritative laboratories in the United States, England, Algiers and Italy as "*Micrococcus*" *melitensis*. We included in this comparative study 32 cultures of *B. abortus* isolated in this country or in England from aborted fetuses or pathologic discharges, or milk of cattle and hogs.

All the cultures were repeatedly plated on glycerol-peptic digest agar and are kept in triplicate sets on the same medium at room temperature. The tests to be recorded have been repeated at least three times, all the strains being tested for the most part simultaneously. A selected number of strains used in the serologic tests reported in the second paper were studied more extensively after rapid transplantation on the same medium for at least 10 to 15 generations. The inoculated tubes were kept sealed with paraffin wax.

MORPHOLOGY

"Micrococcus" melitensis.—The stock cultures designated "*Micrococcus*" *melitensis*, when grown on peptic digest agar or broth with a reaction of P_H 7.2-7.4 for 24 to 36 hours at 37 C., revealed in preparations stained with gentian violet short, stumpy, oval or egg-shaped rods frequently tapered at both ends. Identical smears stained by Gram's method and counter-stained with dilute carbol-fuchsin furnished pictures in which the organisms appeared more coccoid in morphology. This observation is quite in accordance with the findings of Fabyean,⁵ made on *B. abortus*; he noted that carbol-fuchsin accentuated the diameter and gentian violet, the length. In hanging drop preparations the organisms are immotile, noncapsulated and appear

³ Jour. Med. Res., 1913, 29, p. 299.

⁴ Jour. Bacteriol., 1918, 3, p. 193

⁵ Jour. Med. Res., 1912, 26, p. 477.

more like elongated cocci or diplococci. Frequently in the water of condensation or liquid mediums, short chains consisting of from 4 to 10 single, elongated, influenza-like bacillary or stumpy, coccoid elements can be recognized. Strain 2 produces these forms rather frequently; while strains 1, 5, 7, 18, 22 and 26 form single coccoid rods, which are evenly distributed in the stained preparations. Strains 8, 9, 20, 21 and 23 invariably appeared in young cultures as fine small rods in parallel grouping. The individual elements may stain more intensely at both ends and measure from 0.8-1.8 microns in length and from 0.4-0.6 microns in width. The forms most frequently recognized in young cultures on glycerin peptic digest agar, are illustrated in microphotographs 1-6.

It is quite evident that we are unable to recognize the interpretation of Eyre,⁶ who considers these bacillary forms to be staining artefacts. And again the finding of bacilli in 24 hour old cultures on the most suitable mediums with an optimum reaction and oxygen refutes the conception that they are involution forms. We admit, however, the occasional occurrence of a cultural growth after 12 to 18 hours' incubation on suitable solid substratums, which in carbol-fuchsin or thionin preparations consists mainly of coccoid-like elements, indistinguishable from the elements of a young culture of meningococci. A few incomplete tests suggest that definite cyclical changes in the development similar to those described for a variety of organisms by Clark and Ruehl⁷ exist also for the *B. melitensis*. A detailed study of this phase of the problem is in progress.

When stained in thin preparations the organisms of all our strains are gram negative. Repeated tests failed to demonstrate flagella by the method of von Ermengen. Our observations on the morphology of "*Micrococcus*" *melitensis* support, therefore, the finding of Durham,⁸ Galli-Valerio,⁹ Besson,¹⁰ Pollaci,¹¹ and Muir and Ritchie.¹² We therefore concur in the interpretation given by Miss Evans and demand that the generic name "*Bacterium*" be given to the causative organism of undulant or Malta fever.

⁶ Kolle and Wassermann's Handbuch d. pathog. Microorg., 1913, 4, p. 424.

⁷ Jour. Bacteriol. 1919, 4, p. 615.

⁸ Jour. Path. & Bacteriol., 1899, 5, p. 377.

⁹ Centralbl. f. Bakteriologie, I, O, 1904, 35, p. 81.

¹⁰ Practical Bacteriology, London, 1913, p. 475.

¹¹ Centralbl. f. Bakteriologie, I, Ref. 1908, 42, p. 676.

¹² Manual of Bacteriology, 7th Ed., London, 1919, p. 501.

B. abortus.—The morphologic appearance of the various strains of *B. abortus* on the same medium are similar to those of *B. melitensis*. Again, in preparations stained with gentian violet short ovoid or longer rods are demonstrated. The diphtheroid-bacilli-like grouping of the small rods and the indications of granular staining are perhaps more frequently seen in young *B. abortus* cultures, than in those of *B. melitensis*. The length varies between 0.4-2.2 microns and the width between 0.4-0.8 micron. Short chains of coccoid elements are also noted in the water of condensation of young cultures. Recently isolated strains, which are not fully adapted to the new oxygen requirements and the new substratum, appear more coccoid than old, vigorously growing stock cultures. The organisms are always distinctly gram-negative. Microphotographs 7 and 8 illustrate these observations fully (see also, Figs. 1 and 2 on Tafel II, Arb. a. d. k. Gsundhtsamte., 1912, 43, p. 129, and Kolle-Wassermann's Handb. d. pathog. Microog., 1913, 6, p. 299).

In this connection it may appear advisable to recall briefly the various statements relative to the morphologic appearance and the botanical classification of *B. abortus* published in the literature. Preisz¹³ placed the causative organism isolated by him from cases of infectious abortion on account of its irregular staining reaction and its diphtheroid-like grouping with the corynebacteria. It is, however, not unlikely that the organism described by Preisz is not identical with the bacillus of Bang.¹⁴ According to Novak,¹⁵ *B. abortus* resembles the coccobacillus of chicken cholera, and it is therefore grouped with the pasteurella or hemorrhagic septicemia bacilli. Holth¹⁶ considers the organism on "ausgesprochener Kokkobazillus," and Zwick and Zeller¹⁷ noted several strains which possessed a "fast kokken-ähnliches Aussehen," which in turn resembled by dark-field illumination the bipolar bacteria of fowl cholera or swine plague. Fabyean¹⁸ states that "there is some variation in length which in some individuals may be equalled by the diameter, this type suggests a coccus." Our personal observations are therefore fully corroborated by the findings made by other workers. We found it impossible to distinguish *B. melitensis* from *B. abortus* when using cultures with fictitious labels prepared from our stock sets, irrespective of the fact that our constant working with the strains should have impressed on our mind the essential differentiating characteristics. On morphologic grounds the organisms of undulant fever and of infectious abortion of domesticated animals must therefore be considered as identical and must be placed together in the genus bacterium. For reasons to be given in detail in the second paper it is proposed in accordance with the suggestions made by Buchanan¹⁹ of the Committee on Classifications of the Society of American Bacteriologists, that a genus,

¹³ Centrallbl. f. Bakteriologie, 1, O, 1903, 33, p. 190.

¹⁴ Zwick and Zeller, Footnote 17, p. 5.

¹⁵ Ann. de l'Inst. Pasteur, 1908, 22, p. 541.

¹⁶ Ztschr. f. Infektionskrankh. parasitärkrankh. u. Hyg. d. Haustiere, 1911, 10, p. 208.

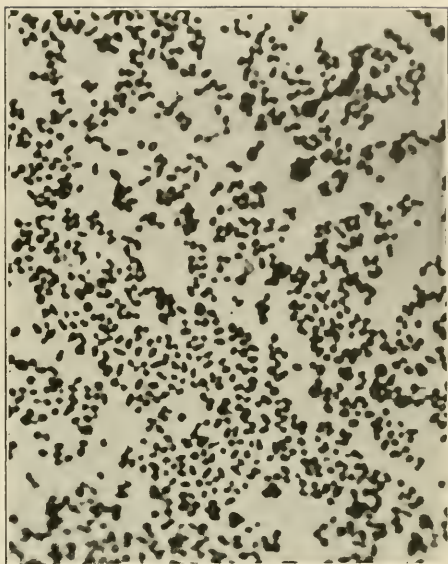
¹⁷ Arb. a. d. Gsundhtsamte, 1912, 43, p. 11.

¹⁸ Jour. Med. Res., 1912, 26, p. 476.

¹⁹ Abstracts Bacteriol., 1918, 2, p. 8.



Strain 7.



Strain 1.

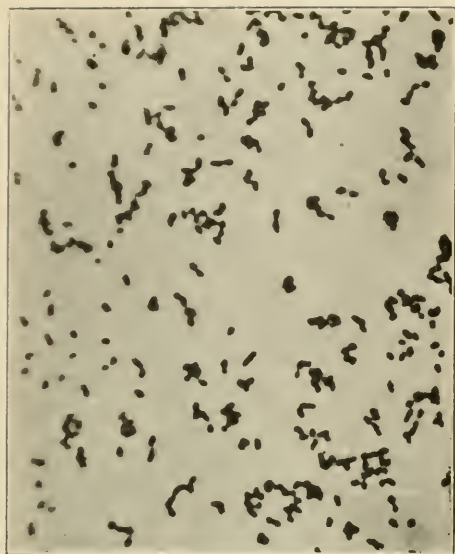


Strain 22.



Strain 18.

Fig. 1.—*B. melitensis*, $\times 1,500$.



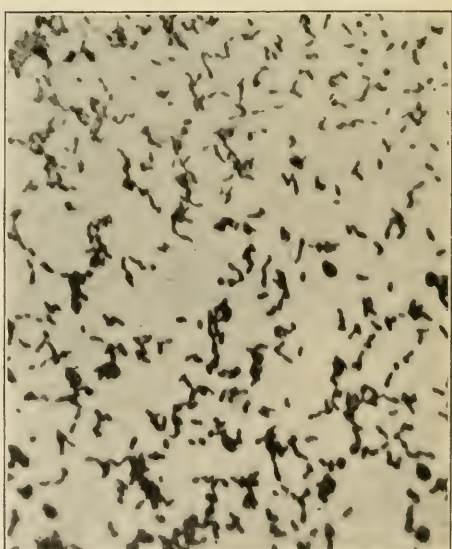
B. melitensis strain 26.



B. melitensis 20.



B. abortus strain 80.



B. abortus 12.

Fig. 2.—*B. melitensis* and *B. abortus*; $\times 1,500$.

for which we propose the name "Brucella," be created in the family Bacteriaceae to separate and to distinguish properly these important pathogenic micro-organisms from the other members of the recognized genus bacterium, which is already overburdened with representatives, which have little in common with the *B. melitensis* and *B. abortus*.

CULTURAL CHARACTERISTICS

The descriptions given by Horrocks,²⁰ Eyre,⁶ and others for the growth of *B. melitensis* and by Bang and Stribolt,² Holth,¹⁶ Zwick and Zeller,¹⁷ and Fabyean,⁵ for that of *B. abortus* on various solid and liquid mediums are fully in accord with our own observations, and it is unnecessary to duplicate the recital of facts. We intend to point out chiefly the slight differences which can be noted and perhaps be chosen to separate on cultural grounds the *B. melitensis* from the *B. abortus*.

It is well known that both organisms grow slowly, even on suitable medium visible growth is not recorded before 36-48 hours' incubation at 37 C. It is furthermore established that primary isolation of *B. abortus* from pathologic material on glycerol or serum agar can only be accomplished successfully by either reducing (Bang and Stribolt, Novak and also Fabyean) or increasing (Holth) the oxygen tension of the atmosphere, in which the organism is placed for artificial cultivation. A similar adaptation for *B. melitensis* has not been reported in the literature. It is not unlikely that the usual isolation of this organism from the blood stream of patients in liquid medium or the milk of goats explains the difference. The peculiar adaptation phenomenon of *B. abortus* to varying oxygen tensions, when first isolated from amniotic fluid or uterine material in general is probably merely the result of its intra-uterine existence as clearly demonstrated by McFadyean and Stockman²¹ and also by Holth. Through the observations of Schroeder and Cotton,²² A. S. Evans²³ and Steck,²⁴ who isolated the *B. abortus* directly from milk in ordinary petri-dishes, even in gelatin medium, and the fact that even prolonged sojourn of the organisms in guinea-pig tissues does not confer this adaptation phenomenon again, it is clearly indicated that the adjustment to definite oxygen tensions is primarily a characteristic for the organism living in the uterine cavity. As we had no access to actual cases of undulant

²⁰ Report of the Commission on Mediterranean Fever, London, 1905, Part 1, p. 5.

²¹ Report of Departmental Committee on Epizootic Abortion, Append. to Part I, Lond. 1909, p. 25.

²² Twenty-Eighth Annual Report of the Bureau of Animal Industry, Dept. of Agricult. 1911, p. 139.

²³ Jour. Infect. Dis., 1916, 18, p. 437.

²⁴ Schweizer Arch. f. Tierheilk., 1918, 60 p. 547.

fever, we are unable to express an opinion concerning the adaptation of *B. melitensis* to varying oxygen tensions. We noted, however, repeatedly that cultures made from guinea-pig spleens, which had been injected with various *B. melitensis* strains 3 to 4 months previous to the time of necropsy, gave only growth in paraffin sealed blood or glycerin peptic digest agar tubes. Plain agar plates or veal infusion agar slants loosely closed with cotton plugs frequently remained sterile. Some tests indicated that the composition of the medium influences considerably the chances of the primary isolation of *B. abortus* and *B. melitensis*. Our pig or beef-liver peptic digest agar²⁵ is admirably suited for this work. Dr. J. Traum of the University of California isolated on this medium in open unsealed slants *B. abortus* directly from the amniotic fluid in the stomach and from the liver of aborted pig's fetuses. The use of "nutrose," "somatose" and beef serum by English experts on Malta fever is repeatedly recorded, and it is not unlikely that these "growth accessory" substances helped in the primary isolation of *B. melitensis* and acted in a similar manner to our digest agar. The majority of our strains were naturally stock cultures, grew therefore abundantly on all culture mediums and appeared fully accustomed to saprophytic life. The three paramelitensis strains 9, 22 and 23 and strain 18 were shy growers when received and even repeated subculturing only slightly enhanced the cultural vigor in comparison with the other strains. We found the optimum reaction of the medium to be a H-ion concentration of P_H 7.2-7.4. Both types of organisms are slightly more alkali than acid tolerant.

The growth on glycerol peptic digest agar or hormone blood-agar plates appears at 37 C. in form of small convex, glistening, pearly-white, droplet-like colonies, which may develop into colonies of from 2-8 millimeters in diameter. The paramelitensis strains 22 and 23 produced sometimes rather granular, dull, comparatively flat colonies; this phenomenon was particularly marked on dry plates. *B. abortus* colonies cannot be distinguished from those of *B. melitensis*.

On agar slants a fine granular film appears in from 24-36 hours; after 3 days a slight brownish tinge changes the moist, well defined growth. Development continues on peptic digest agar for weeks, even at room temperature, until a stringy, greasy, rather thick layer covers the inoculated agar surface. This growth remains amber or honey-like yellow or perhaps caramel-like brownish (see Fig. 1, Plate 28, twenty-

²⁵ Stickel and Meyer, Jour. Infect. Dis., 1918, 23, p. 68.

eighth annual report of the Bureau A, U. S. Dept. Agri.), for *B. abortus* strains even after six weeks' incubation. *B. melitensis* strains 1, 2, 3, 5, 7, 8, 19, 20, 21, 23 and 24, however, changed their growth to a deep chocolate or dirty chestnut brown, some even to a dull ebony black. This pigmentation of the bacterial layer is usually more marked at the upper portion of the slant and may be accompanied by a slight or pronounced darkening of the agar substratum. Intensive dark pigmentation is regularly observed with the cultures of *B. melitensis* mentioned and differentiates these strains distinctly from all our *B. abortus* strains. The time of incubation to produce this pigmentation is not constant and may vary from 8 to 30 days. It is, however, emphasized that a number of *B. melitensis* cultures (4, 6, 9, 10, 11, 18, 25, and 27), which must be classified serologically as typical strains, have failed to produce a darker pigment than on *B. abortus* cultures and differ therefore in no respect from the latter. Crystals, which are probably due to the increasing alkalinity of the medium, were observed only after two weeks' incubation in our digest agar mediums. In veal infusion agar they may appear on the sixth to tenth day of incubation. Agar shake cultures of all strains fail to show a zone of growth as mentioned by a number of writers; there is a thick growth on the surface, which may also extend slightly beneath the surface.

In gelatin, all our strongly pigment-producing *B. melitensis* strains developed dark brownish granular colonies after incubation of from 10 to 30 days. The *B. abortus* strains acted similarly. The medium was never liquefied.

In veal infusion or digest broth a slight initial turbidity, which is followed by a gradual clearing and by a stringy, tenacious sediment, occasionally with a slight pellicle or ring formation, was noted for all the strains studied after 5 to 10 days' incubation. Our *B. melitensis* strains 9, 18, 22, 33, and *B. abortus* cultures 5, 12, 13, and 19 produced a scaly, powdery sediment with little or no turbidity of the supernatant broth medium.

Cultures on potatoes may give varying results, depending on the age of the tuber and its reaction. On properly chosen, slightly alkaline, moist potato-cylinders the behavior of the majority of our *B. melitensis* strains is in some respect characteristic. Inoculated from a broth culture or the water of condensation, the visible growth was always distinctly amber yellowish or even brownish after 5 to 6 days' incubation. The following strains behaved in this manner: 1, 2, 3, 4, 5, 6, 7,

9, 10, 11, 19, 20, 21, 22, 23, 24, 25, 26 and 27. *B. abortus* strains, however, cultivated simultaneously on the same medium and in the same manner, showed only a faintly yellowish hue. After 3 to 4 weeks of incubation, they may show the well-known glanders bacillus-like appearance (McFadyean and Stockman²⁶). At this period the *B. melitensis* strains mentioned are already deep brownish. Very old *melitensis* cultures demonstrate a more intense pigmentation of the bacterial growth and marked brownish discoloration of the potato itself in contrast to the generally light coloring of that of *B. abortus*. Variations in the shading of the color among the latter strains are not uncommon and again the *B. melitensis* cultures 8 and 18 behaved, when repeatedly tested on potatoes, like the *B. abortus* strains.

Bromcresol purple goat's milk in fermentation tubes is turned slightly alkaline after 5 to 10 days' incubation at 37 C. in the open arm by all the strains tested. The H-ion concentration decreases from PH 6.6-7.2-7.4. Litmus milk remains unchanged or turns slightly deeper blue in the open arm. Fresh goat's milk with a layer of cream and bromcresol purple as an indicator shows no visible changes even when incubated for three months. In goat's milk litmus whey, the titrable alkalinity of both the *B. melitensis* and *B. abortus* strains varies after 10 days' incubation between 0.2 and 0.5 per cent. of a normal HCl solution. The differences in the final reaction are merely the result of differences in the rate of multiplication of the various strains. Poorly growing *B. melitensis* and *B. abortus* strains produce a small amount of alkali. The absence of changes recorded in the sterile goat's milk stratified with the cream suggests that the alkaline reaction is caused primarily by the oxidation of the salts of citric acid to alkaline carbonates as recently discussed by Ayers and Rupp.²⁷

BIOCHEMICAL REACTIONS

Hiss' serum-peptone-pheno-sulphonephthalein-water, containing 1 per cent. of levulose, galactose, maltose, saccharose, raffinose, mannite, dulcite or inulin are not fermented by the representatives of the genus "*Brucella*." In glucose and lactose-peptone-phosphate-broth an alkaline reaction develops after 5 to 20 days' incubation at 37 C. The H-ion concentration decreases from PH 6.8 to 7.6 and to 7.8. This reduction, already observed by Eyre and enhanced by Evans, is con-

²⁶ Report of Departmental Committee on Epizootic Abortion, Append. to Part I, London, 1909, p. 4.

²⁷ Jour. Infec. Dis., 1918, 23, p. 188.

stant for all our strains, when final determinations are made after the twentieth day of incubation. Vigorously growing strains as a rule produce this alkaline reaction in a shorter time interval than the poorly growing types (for example *B. melitensis* 18, 22, 23 and the *B. abortus* 3, 10, 15, etc.). Irrespective of the initial H-ion concentration, 16 strains of the 21 *B. melitensis* studied produced a reduction equal to a P_H of 0.6-0.8, two strains of 0.9, one of 1.0 and two of 0.5. Of 20 *B. abortus* cultures tested, the reduction was: 18 a P_H of 0.7-0.8, and two of 0.9. This important and characteristic reaction emphasized by Miss Evans is therefore confirmed by our tests.

Indol is not produced in Difco-peptone solutions by any of our strains. Only *B. melitensis* strains 10 and 24 and *B. abortus* 80, 8, 32, 33 and 38 gave reactions in nitrate broth, which could be interpreted as indicating the presence of nitrites. Neither Horrocks nor Eyre for *B. melitensis* nor Fabyean for *B. abortus* were able to demonstrate a true reduction of nitrates to nitrites.

Neutral red and lead acetate agar give a slight growth with absence of a reduction of the dye or the chemical.

Following the suggestion of Miss Evans, our cultures were also tested for the production of ammonia in asparagin and urea containing mediums. All our strains of *B. melitensis* and *B. abortus* decomposed urea. *B. melitensis* strains 7, 9, 10 and 24 and *B. abortus* 80, 10, 34 and 40 produced a marked amount of ammonia, about equal to one mgm. in 20 c c of medium. On the other hand, the decomposition of asparagin was irregular and in comparison with the one in urea rather slight for most of the *B. melitensis* strains. In the only complete series in which all the actively growing *B. abortus* strains were tested simultaneously either no reaction or indefinite changes were recorded with Nessler's reagent. The following *B. melitensis* strains decomposed asparagin and gave a distinct ammonia reaction: *B. melitensis* 7, 9, 11, 23, 24, 25 and 26. Even vigorously growing strains of the genus "*Brucella*" may therefore fail to register ammonia production in asparagin solutions.

The viability of the cultures of *B. abortus* and *B. melitensis* in sealed tubes protected from desiccation and kept at a uniform temperature (18-22 C.) Eyre,⁶ Mohler, and Traum²⁸ is well known. We were successful in obtaining viable cultures from agar slants of all the

²⁸ Twenty-Eighth Annual Report of the Bureau of Animal Industry, Department of Agriculture, 1911, p. 154.

strains which had remained unopened at room temperature for 6 and 10 months, respectively, after inoculation.

CONCLUSIONS

A comparative study of 21 cultures of so-called "Micrococcus" *melitensis* obtained from various sections of the world and of 32 cultures of *B. abortus* (Bang) isolated in this country and England justifies the following conclusions:

The causative organism of undulant fever of man and of Malta fever of goats cannot be distinguished morphologically or biochemically from the organism responsible for infectious abortion in domesticated animals.

So-called "Micrococcus" *melitensis* appears in young cultures as a short rod and should therefore be designated as *Bacterium melitensis*.

The pigment production of the majority of actively growing *B. melitensis* strains on glycerol peptic digest agar and on alkaline potato cylinders after 5 days' incubation is more intense than with the strains of *B. abortus*.

Both *B. melitensis* and *B. abortus* cultures produce after 20 days' incubation in glucose and lactose broth an alkaline reaction and a characteristic reduction of the H-ion concentration equal to about 0.6 to 1.0 P_H.

H₀. π

Principles in Serologic Grouping of *B. Abortus*
and *B. Melitensis*. Correlation Between
Absorption and Agglutination Tests

Studies on the Genus *Brucella* Nov. Gen. II.

M. L. FEUSIER
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PRINCIPLES IN SEROLOGIC GROUPING OF *B. ABORTUS* AND *B. MELITENSIS*. CORRELATION BETWEEN ABSORPTION AND AGGLUTINA- TION TESTS

STUDIES ON THE GENUS *BRUCELLA* NOV. GEN. II

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San Francisco*

A series of absorption tests, carried out with *B. abortus* and *B. melitensis* antiserums, resulted in a definite grouping of *B. abortus* and *B. melitensis* strains. A similar grouping ensued from a series of agglutination tests, in which eleven antiserums from rabbits, a monkey and a guinea-pig were used. The correlation, under diverse circumstances between the two sets of groupings, permits the inference that certain immutable laws govern serologic reactions, and although these laws remain elusive, we have been impressed by certain important principles involved in grouping.

Some preliminary tests with *B. abortus* and *B. melitensis* antiserums disclosed marked variations in the agglutinability of a number of authentic melitensis strains. In several melitensis antiserums, for example, the range of variability was so great that certain strains appeared to have little in common. Nevertheless, a relationship was apparent in that the strains of high titer in one antiserum perhaps group themselves as low titer strains in another antiserum. Thus while the variability was not identical, it was at least constant for the strains concerned. This suggested that definite principles might govern these variations. In the hope of elucidating these principles and of thus establishing the relationship among *B. melitensis* strains and their relationship to *B. abortus* strains, absorption tests were undertaken with *abortus* and *melitensis* antiserums. These experiments resulted in a four-fold grouping of the strains concerned.

PRELIMINARY DISCUSSION ON ANTIBODIES

The creation of antibodies is explained on the hypothesis that the bacterium consists of one or more components, each producing its specific agglutinins during the process of immunization. Biologically

allied species may possess one or more components in common, hence the phenomenon of group or coagglutination. Castellani demonstrated that by saturating the antiserum with an emulsion of the coagglutinating organism, all these coagglutinins could be removed, leaving the specific agglutinins of the homologous strains intact. It was formerly believed that an antiserum could be exhausted of the specific agglutinins only by saturation with the homologous strain, but both Kruse and the co-workers, Andrewes and Inman, in their study of the serological races of the Flexner dysentery group, found that this was not the case. As the latter workers pointed out, "Of two strains A and B, A may be able completely to exhaust serum B, but B may be unable to do the same for serum A." Our own experience corroborates this point, and we have been able further to specify the condition under which occurs the phenomenon of a nonhomologous strain exhausting an antiserum, namely, when the two strains belong to the same serologic group, but are not identical, as will be demonstrated later.

In comparison to the stress laid on the structure of the bacterium in evoking the formation of antibodies, too little emphasis has been placed on the complexity of the serum itself. It is as if we mixed an acid in an alkaline solution and were to explain the process of neutralization by the composition of the acid regardless of the composition of the alkali. We find, for example, that the same bacterium may evoke the formation of quantities of antibodies in the serum of one species of animal, while it evokes but a feeble amount in a serum of another species;¹ yet we evade all responsibility of attempting to unravel the mystery from the point of view of the serum. We appease our curiosity with some selfevident platitude, such as the individuality of the animal, and thus veneer our ignorance with a gloss of logic. Why this individuality? Until serologic workers direct their investigations to unraveling the complexity of the serum, we cannot hope to fathom the marvelous mechanism of serologic reactions, nor can we offer any adequate explanation why a fourfold division of bacterial species results repeatedly from serologic classifications. Meningococcus (Gordon), pneumococcus (Cole and associates), tetanus (Tullock), Flexner dysentery (Andrewes and Inman),² typhoid (Weiss

¹ For example, one of our strains produced agglutinins to 1:20,000 in a monkey, and 1:2,000 in a rabbit.

² According to Andrewes and Inman, their "Y" or fifth race contains no specific antigenic component but presents a mixture of the component of the other four races. They say "we have failed fully to solve the antigenic structure of the 'Y' races. All that we have been able to do has been to obtain evidence of the presence in the race of most or all of the antigenic components which we have termed, V, W, X, and Z." (p. 36) Ref. 1.

and Hooker), streptococci (Dochez, Avery and Lancefield), influenza (Small-Dickson), and our own work on *abortus-melitensis* are included in this fourfold division. Is it not significant that the isolysins and iso-agglutinins of the human race also fall into four groups?

PRELIMINARY DISCUSSION ON ABSORPTIONS

In all our absorption tests we have proceeded on the principle that incomplete absorption of the nonspecific agglutinins, while it may yield interesting data, cannot establish definite laws. Unless the antiserum is absorbed to extinction of its nonspecific agglutinins, no subsequent reaction can be classed as distinctly specific. As a control, our absorbed serum was always tested with the absorbing strain along with our entire series of experimental organisms. If any agglutinins remained for the absorbing strain, the test was discarded. In practice we found it helpful to make a preliminary test with the absorbing strain, and if all of its agglutinins had not been removed the serum was reabsorbed. When the limit of extinction is reached, no further saturation with a nonspecific strain will effect a reduction in the titer of the serum for the specific agglutinins. On the other hand, as we have observed, an incomplete absorption, on becoming complete, may further reduce the specific titer 50%. It seems of vital importance, therefore, if we are to class residual agglutinins as specific that the nonspecific agglutinins should be absorbed to extinction.

Logically, the term specific is relative to the absorbing strain—for different bacteria may absorb different amounts and kinds, and in each case we term the residual agglutinins "specific." It may be possible that the definitely specific agglutinins of a bacterium can be measured only by successive saturations of its antiserum with different bacteria, each capable of removing quantitatively and qualitatively its own coagglutinins. But even then, there would be a minimum of residual agglutinins beyond which no bacterium except the homologous strain, or one of the same group, could exhaust the antiserum. We have not performed such tests and we use the term specific in its accepted sense of residual agglutinins after absorption with a nonhomologous strain.

TECHNIC

Antigens.—The strains were grown on peptic digest agar, the growth washed off with a few cc of formalinized salt solution and stored in the ice chest as stock emulsions. To 100 cc of formalinized salt solution the necessary amount of emulsion was added to match a standard in capacity containing about one

and one-half billion organisms. The same amount was added in each case whenever it became necessary to replenish the antigens. Although we use light suspensions throughout the experiments, we have since made use of opaque suspensions which have yielded excellent results. All agglutination readings were recorded after 18 hours in the incubator.

Method of Absorption.—The antiserum was diluted 1:10 with salt solution. An equal quantity of an emulsion of the absorbing antigen was added, making a dilution of 1:20. The tube was left 2 hours in the incubator and overnight in the ice chest, after which it was centrifuged one hour. The clear serum was decanted and a preliminary test made with the absorbing strain, to determine whether all of its agglutinins had been removed. If not, the serum was reabsorbed, using packed cells of the antigen in order not to alter the dilution of 1:20. The following day the clear serum was tested for agglutinins with 14 of our experimental strains, including the control. Thus we obtained evidence of relationship, not only between the absorbing and the homologous strains, but also for a number of other strains. Grouping at once became apparent.

Antisera.—Although seven antisera were employed in the course of our absorption investigations which covered over 400 tests, we submit the data from two of these, these two having been absorbed systematically by the greatest number of strains. Table 1 also contains the data of absorption tests from monkey antiserum. The magnitude of the task prevented us from pushing the absorption tests to completion in all the antisera, but about 100 selected tests convinced us that the results paralleled each other. The antisera of the classification submitted were made by immunizing rabbits, with both dead and living cultures, one with *B. abortus* 80, and the other with *B. melitensis* 7, these being our classical stock strains of *B. abortus* and *B. melitensis*.

RESULTS AND RECORDS OF ABSORPTION TESTS

It is quite evident that by absorbing an antiserum with one strain and then testing it with a number of other strains we may obtain one of three results. The coagglutinins may have been removed for all the remaining strains, thus giving a negative reaction throughout; they may have been removed for none of the remaining strains, thus giving a positive reaction throughout; or they may have been removed for some and not for others, thus giving a negative reaction for the former and a positive reaction for the latter. In the first and second cases we obtain no information as to grouping, all strains having followed an identical course. In the third case, however, a division into two groups is apparent. Let us assume, for example, that a certain serum absorbed by a certain strain was then positive for A, B and C, and negative for X, Y and Z, thus giving us two groups. Now another serum absorbed by the same strain, or if we choose the same serum absorbed by another strain, may leave A, B and Z negative, while X, Y and C are positive. We now have four groups. A and B have acted identically

throughout, and X and Y have clung together; C has fallen into a group by itself, and Z has done likewise. A third absorption with another serum or another strain may leave A and Y positive, and B, C, X and Z negative. We would now have as many groups as strains, for no two would have followed an identical course throughout.

Apparently this differentiation into groups is based on affinity for the same agglutinins. So long as two strains continue to follow parallel courses under various conditions, they are exhibiting like properties and may be assumed to possess similar components; for when the coagglutinins are absorbed from an antiserum for one of the strains, they are absorbed for the other; when the coagglutinins remain for the one, they likewise remain for the other. If this uniform behavior continues throughout a series of different types of tests, it is reasonably evident that the strains have a preponderance of something in common. Since they constantly react uniformly they naturally group themselves in the same categories.

At a special stage in our work, we became conscious of this group affiliation. Certain strains were exhibiting identical reactions (qualitatively) regardless of the antiserum or the absorbing strain used. There was, it is true, a quantitative difference—the titer when positive was higher or lower—but qualitatively they reacted in a uniform manner—their coagglutinins were either absorbed or were not absorbed under the same conditions. By checking the results, we found that the fourteen experimental strains fell into four groups with the greatest number in group 2.

These groups were as follows:

Group 1: *Melitensis*, 20; *abortus*, 80.

Group 2: *Melitensis*, 18, 19, 21, 2, 6, 8, 11, 655, 10.

Group 3: *Melitensis*, 7.

Group 4: *Paramelitensis*, 22; *paramelitensis*, 23.

Other strains tested irregularly distributed themselves in the various groups, the *abortus* strains invariably falling in group 1. Table 1 represents a portion of the data from which these groups were compiled. The results are expressed qualitatively (not quantitatively). The experimental strains are in the extreme left column and their reactions are to be read in a horizontal line. The strain and antiserum used in the absorption test are indicated at the top of the column and at the bottom, the groups to which they belong.

In endeavoring to analyze the principles involved in this grouping our data (as may be seen from the table) indicated that the strains

of one group could not exhaust the antiserum of another group; for example, no strain of groups 2, 3 or 4 could exhaust the antiserum of group 1. The antiserum of group 1 could be exhausted only by its homologous strain or by some other strain of group 1; This suggests that there is a specific component in each group which differentiates it from all other groups. On the other hand, it is apparently a common property for members within a group to exhaust the antiserum one

TABLE 1

QUALITATIVE RECORDS OF ABSORPTION TESTS REPRESENTING THE FOUR GROUPS OF *B. ABORTUS* AND *B. MELITENSIS*

Agglutinated with	Rabbit								
	Anti-meliten-sis 7 Absorbed with Meliten-sis 7	Anti-abor-tus 80 Absorbed with Meliten-sis 7	Anti-meliten-sis 7 Absorbed with Meliten-sis 11	Anti-abor-tus 80 Absorbed with Meliten-sis 11	Anti-meliten-sis 7 Absorbed with Meliten-sis 18	Anti-abor-tus 80 Absorbed with Meliten-sis 18	Anti-meliten-sis 7 Absorbed with Meliten-sis 19	Anti-abor-tus 80 Absorbed with Meliten-sis 19	Anti-meliten-sis 7 Absorbed with Meliten-sis 21
telitensis 2.....	0	+	0	0	0	0	+	0	0
telitensis 6.....	0	+	0	+	0	+	0	+	0
telitensis 7.....	0	0	+	0	+	0	+	0	+
telitensis 8.....	0	+	0	+	0	+	0	+	0
telitensis 10.....	0	+	0	0	0	Not tested	0	0	0
telitensis 11.....	0	+	0	0	0	+	0	0	0
telitensis 18.....	0	+	0	0	0	0	0	0	0
telitensis 19.....	0	+	0	0	0	+	0	0	0
telitensis 20.....	0	+	0	+	0	+	0	+	0
telitensis 21.....	0	+	0	+	0	+	0	0	0
telitensis 22.....	0	0	+	0	+	0	+	0	+
telitensis 23.....	0	0	+	0	+	0	+	0	+
telitensis 655.....	0	+	0	0	0	+	0	0	0
abortus 80.....	0	+	0	+	0	+	0	+	0
	Group 3 absorbed with group 3	Group 1 absorbed with group 3	Group 3 absorbed with group 2	Group 1 absorbed with group 2	Group 3 absorbed with group 2	Group 1 absorbed with group 2	Group 3 absorbed with group 2	Group 1 absorbed with group 2	Group 3 absorbed with group 2

+ signifies agglutination after absorption.
 0 signifies no agglutination after absorption.
 ± signifies indistinct reaction.

for another without a reciprocal exhaustion taking place. Indeed in our limited investigations along this line, it was the prevailing case. We are not prepared to state that it is an obligatory relationship for one strain to be able to exhaust the antiserum of another strain in the same group, but we suspect that such may be the case. This implies a close relationship among the allied strains of the same group.

The second point revealed was that if a strain exhausted an antiserum of its coagglutinins for some strain in another group, it exhausted the coagglutinins for all strains in that group under the same absorption conditions; that is, the action was uniform (qualita-

tively) on the entire group. If the reaction were positive, the same principle applied. To illustrate the foregoing points, let us glance at the above grouping and suppose that strain 18 removed from antiserum 7 the coagglutinins for strain 20 (group 1), but not for strain 22 (group 4). Then strain 18 also removes the coagglutinins from antiserum 7 for strain 80 and for all other members of this group, but does not remove them for strain 23 or for any other members of this

TABLE 1—Continued

QUALITATIVE RECORDS OF ABSORPTION TESTS REPRESENTING THE FOUR GROUPS OF B. ABORTUS AND B. MELITENSIS

Antiserums						Monkey Antiserum					
Anti-abortus 80 Absorbed with Meliten- sis 21	Anti-meliten- sis 7 Absorbed with Paramel- itensis 22	Anti-abortus 80 Absorbed with Paramel- itensis 22	Anti-meliten- sis 7 Absorbed with Paramel- itensis 23	Anti-abortus 80 Absorbed with Paramel- itensis 23	Anti-meliten- sis 7 Absorbed with Meliten- sis 20	Anti-abortus 80 Absorbed with Meliten- sis 20	Anti-meliten- sis 7 Absorbed with Abor- tus 80	Anti-abortus 80 Absorbed with Abor- tus 80	Anti-meliten- sis 655 Absorbed with meliten- sis 7	Anti-meliten- sis 655 Absorbed with Abor- tus 80	Anti-meliten- sis 655 Absorbed with Meliten- sis 655
0	+	+	+	+	±	0	±	0	+	Not tested	0
+	Not tested	+	Not tested	+	±	0	±	0	+	+	0
0	+	?	+	0	+	0	+	0	0	0	0
+	+	+	+	+	±	0	±	0	+	+	0
+	Not tested	+	Not tested	+	±	0	±	0	+	Not tested	0
+	+	+	+	+	±	0	±	0	+	+	0
+	+	Not tested	Not tested	+	±	0	±	0	+	+	0
0	+	+	+	+	±	0	±	0	+	+	0
+	+	+	+	+	0	0	0	0	+	0	0
0	+	+	+	+	±	0	±	0	+	+	0
0	0	0	0	0	+	0	+	0	0	0	0
0	0	0	0	0	+	0	+	0	0	0	0
+	+	+	+	+	±	0	±	0	+	+	0
+	+	+	+	+	0	0	0	0	+	0	0
Group 1 absorbed with group 2	Group 3 absorbed with group 4	Group 1 absorbed with group 4	Group 3 absorbed with group 4	Group 1 absorbed with group 4	Group 3 absorbed with group 1	Group 1 absorbed with group 1	Group 3 absorbed with group 1	Group 1 absorbed with group 1	Group 2 absorbed with group 3	Group 2 absorbed with group 1	Group 2 absorbed with group 2

group. We see, then, that a strain acts in a uniform manner on every member in another group, under the same absorption conditions.

We observed further (but we are not prepared to state this as a universal fact) that all strains in one group were likely to act in the same manner (qualitatively) on all members in another group when absorbed from the same antiserum. For example, continuing the above illustration, our data revealed that strains 19, 21 and 11 (same group as 18) also removed the coagglutinins from antiserum 7 for strains 20 and 80, but did not remove them for strains 22 and 23. The same principle asserted itself when five strains of group 2 were absorbed from group 1 antiserum. Here the reaction was positive for strains 20 and 80, and negative for strains 22 and 23 in all 5 cases.

We may state this tentatively as follows: All strains in one group tend to act in the same manner (qualitatively) on all strains in another group, when absorbed from the same antiserum. If this is true, there is a uniform action of group on group, which is more than our principle advocates, namely, the uniform action of each strain on the entire group.

Immediately a third principle manifested itself as an amendment to the preceding. Occasionally a group did not act in unison, but analysis revealed the definite condition under which this deviation occurred; namely, an absorbing strain might act in an irregular manner on members of its own group, thus bringing out their individual differences. For example, strain 18 when absorbed from antiserum 80 removed the coagglutinins for itself and for strain 2, but did not remove them for the remaining strains of the same group; that is, we have a mixture of positive and negative reactions for members of the same group when subjected to the same absorption conditions. It may be that the difference among members within a group is purely a quantitative one, that each possesses a preponderance of the specific agglutinins but varying amounts of the foreign coagglutinins, and this difference becomes manifest only when one of the group acts as the absorbing agent in removing the coagglutinins. It must be borne in mind that while a group absorbs irregularly for its own members the whole group is acted on uniformly by members of another group.

So far as we carried our experiments, we found no deviation from these three principles. We attempted to check our results by the following test: One of the workers planted from his own private stock 6 of the experimental strains and gave them to the other worker under fictitious lettering. In all 6 cases the strains were assigned to their proper groups and in 4 cases the exact organism was located. The latter point, however, is beyond the scope of our work. We cannot scientifically separate one strain from another in the same group, and the ability to do so is merely temporary and due to that intangible evidence which constant handling of a strain brings to a worker.

The three principles enunciated above may be briefly summarized:

1. An antiserum cannot be exhausted by strains of another group. It is always exhausted by its homologous strain and may be exhausted by other members of the same group.
2. A strain acts in a uniform manner (qualitatively) on all strains in another group under the same absorption conditions. This uniform action constitutes the basis for group affiliation.

3. Strains within the same group do not necessarily act in a uniform manner on one another under the same absorption conditions. This constitutes the basis for individual differentiation.

The degree of demarkation between any two groups is far from uniform. There is a wide difference between groups 1 and 3 and between groups 1 and 4, but the relationship between groups 1 and 2 is exceedingly close, and generally they follow identical courses only to be separated when some specific element comes into play. Frequently the strains of these groups interagglutinate to their full titers. Group 2 appears to be a transition between groups 1 and 3. It bridges their differences; it is related to both, but it is sharply separated from group 4. Group 3 may be taken as the type of the classic melitensis strain, from a serologic standpoint. Its agglutinins are in a large measure specific, and it is clearly defined from other types. Group 4 embraces strains of low agglutinability and of low antibody producing powers. They are fairly agglutinable in some antisera if living suspensions are used, but all our work was carried out with formalinized suspensions. They appear to have few agglutinins in common with groups 1 and 2; they have a more pronounced absorbing effect in group 3 than their agglutinating titer might intimate. But agglutinating titer is not a criterion for absorbing titer. In comparing the percentage agglutinating with the percentage absorbing titer for the different groups, it was found that the percentage absorbing power of a group was frequently in excess of its percentage agglutinating power. The reverse was rare. On the whole, group 3 appears to be the pivot from which the strains radiate in one direction toward the abortus group, and in the other direction toward the highly specific paramelitensis group.

It has been stated that whenever *B. abortus* strains were tested they fell in group 1. We carried but one abortus strain, number 80, throughout all the tests. Into the same group fell melitensis 20. Under all absorption conditions these two strains followed identical courses except that there was a quantitative difference in titer. Melitensis 20 could exhaust the antiserum of *B. abortus* 80 and toward the end of our work when we prepared an antiserum for melitensis 20, we found that it could be depleted by *B. abortus* 80, thus giving a reciprocal exhaustion. We then reduced the absorbed dilution to 1:10, and 4 other *B. abortus* strains were now absorbed from melitensis 20 anti-

serum. All 4 exhausted it. One of these strains had been carried through about half the absorption test. We now found that melitensis 20 could exhaust its antiserum. Thus we had two *B. abortus* strains which could exhaust the antiserum of melitensis 20 and whose antiserum melitensis 20 could exhaust. Nevertheless, the 3 strains are not identical, so far as their histories go. Their titers, though approximate, are not identical in all antisera. Their coagglutinins are not equally removed quantitatively by other strains. They grow differently both as regards speed and abundance. We are, therefore, forced to conclude that reciprocal exhaustion in dilutions as low as 1:10 is no criterion for identity of strains. This sounds illogical, but it does not exclude the possibility that specificity may be demonstrable in dilutions of 1:2 or 1:5. All that we conclude from these reciprocal absorption tests is the close relationship between *B. abortus* and one type of *B. melitensis* strains. What then is specificity? The fact that a nonhomologous strain can exhaust an antiserum casts a doubt on individual specificity as the exclusive possession of a single bacterium. It would appear that no bacterium is an isolated entity, all of its agglutinins provoked by immunization, are shared by some members in its group and out of its group. Each group may possess a separate primary attribute, but not each bacterium, and the sum total of these primary attributes constitutes the race; for example, the race of typhosus, of dysenteriae, of pneumococcus. The individuality of a bacterium would then appear to consist in its proportional share of the agglutinins of its race—the primary group agglutinins predominating—rather than in the possession of a specificity exclusively its own.

Andrewes and Inman,³ in their masterly article on the Flexner dysentery types, used a quantitative method in their absorption tests. They determined the number of bacteria in their absorbing emulsions and diluted these for two absorbing doses, one containing approximately 1,000 million organism, and the other from 20,000 to 30,000 million organisms. They advocate a quantitative method. We have not been convinced of the advantage in determining the number of organisms in the absorbing dose—except its interest from the experimental point of view. It would be vexatious in routine work even if emulsions were kept in stock, and moreover its result might be fallacious. If our principle is correct that the absorbing strain must

³ Medical Research Committee Special Report No. 42, 1919.

remove all the coagglutinins it is capable of absorbing—that is, to extinction of itself—there could be no fixed doses for any one strain, because its absorbing capacity varies with the potency of the serum and its relationship thereto.

When dense doses are required there seems to be less hindrance to the progress of the reaction, if absorbed fractionally. This, however, is not obligatory.

GENERIC CLASSIFICATION

The American Committee on the Classification of Bacterial Types⁴ decided that “*B. abortus* may for the present be left in this genus (*Bacterium*) in spite of its peculiar oxygen relations.” The genus *Bacterium* of the *Bacteriaceae* family constitutes the colon-typhoid-dysentery group. It would seem that this genus is already encumbered with sufficiently diversified types without the addition of *B. abortus*.

If in reality a classification is a scheme destined to convey some adequate idea of mutual relationships, should not its genera be so apportioned that each genus may be narrowed to a type, embracing individuals with fairly limited common characteristics and common differentiations from other types; thus, one genus should not include organisms with such widely varied specificity as *B. coli*, *B. typhosus*, *B. dysenteriae* and *B. abortus*, although all these would still be united in a common family. If, as is the case in the above genus, the “species” is left as the sole vehicle for differentiation (for the term subgenus is a useless encumbrance), a classification becomes an empty nomenclature, a mere vocabulary with which the sophisticated student may terrify the uninitiated scholar.

We advocate, therefore, that *B. abortus* be removed from the genus *Bacterium*, which includes the colon, typhoid and dysentery organisms, and we suggest that the *abortus melitensis* group be given separate rank as the genus “*Brucella*” (from Bruce who isolated the original *melitensis* organism, later identified by Nègre and Raynaud, as *Micrococcus paramelitensis*.⁵) The 4 groups as above formulated would then each embrace a number of allied species, and if it became expedient to establish subgroups, they would probably range themselves as varieties of some species.

⁴ Jour. Bacteriol., 1917, 2, p. 546.

⁵ Compt. rend. Soc. de biol., 1912, 72, p. 791 and 105.

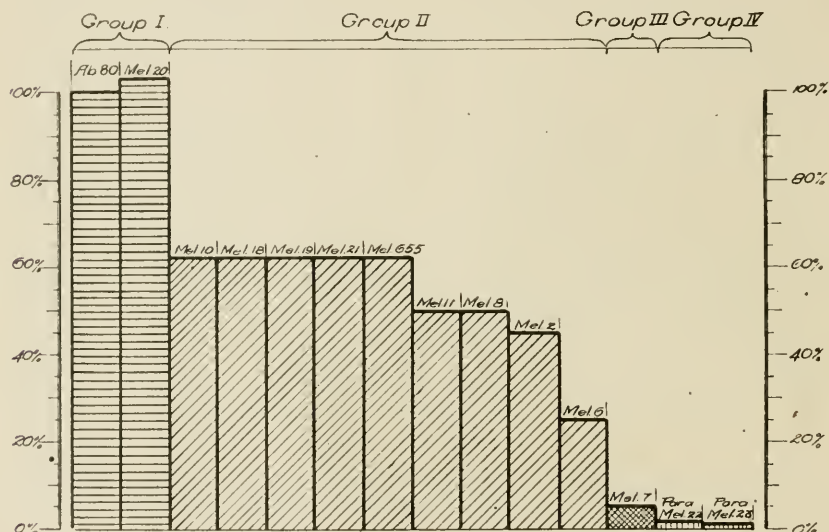
RESULTS AND RECORDS OF AGGLUTINATION TESTS

The experimental strains were agglutinated in 1 monkey, 1 guinea-pig and 9 rabbit antisera. All 4 groups were represented by these 11 antisera. We also tested the strains in the serums of 2 cows and 3 hogs suffering from natural abortion disease. Separate references will be made to these tests.

In addition to the experimental strains used in the absorption tests, we carried 15 other *B. melitensis* strains through all the tests and 35 *B. abortus* strains were agglutinated in 6 of the antisera.

B. abortus and *B. melitensis* unlike *B. typhosus*, for example, are not strong antibody producers. Our *B. abortus* antisera ranged from 1:2,000 to 1:4,000 and our *B. melitensis* antisera approximated 1:2,000 except that

Chart 1.—Agglutinogenesis of group 1 antiserum. Columns of like marking represent different strains of the same group.



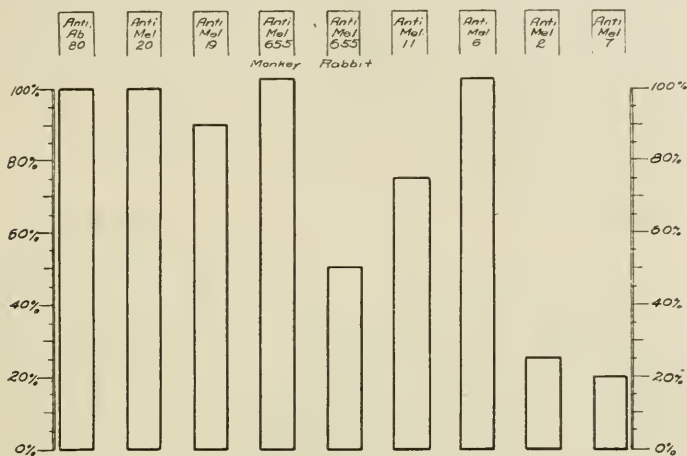
Abortus 80 antiserum (group 1). Percentage agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

of the monkey which was active in a dilution of 1:20,000 and the so-called paramelitensis of group 4 which did not yield an antiserum beyond 1:200 in either a rabbit or a guinea-pig.

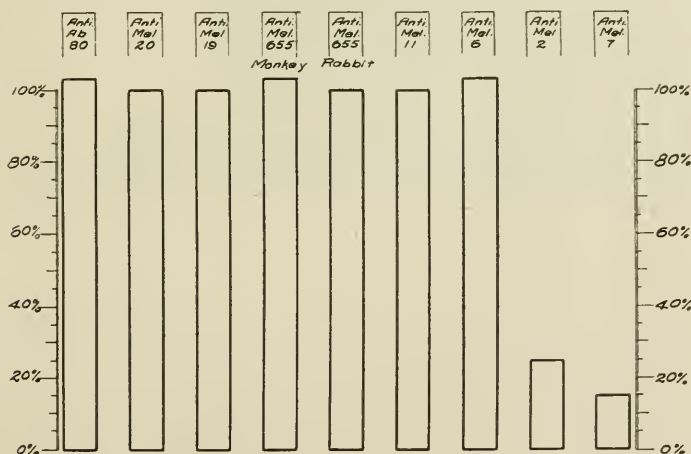
In expressing our results graphically, we have adopted the column and percentage method used by Andrewes and Inman.⁸ As these workers pointed out, actual figures are not comparable owing to the different titers of various antisera, whereas results are readily comparable if expressed as percentages of the titers for the homologous strains. The titer for the homologous organism is taken as 100% and the proportional titers for others are expressed as percentages of this. Thus, if a strain reacts to 1,000 in an antiserum which flocculates the homologous organism to 2,000, the former's titer is expressed as 50%. Occasionally a strain reacts beyond the titer of the homologous organism, in which case the percentage is expressed above the 100 mark.

In presenting the results which follow we shall discuss first, the action of the antisera of each group on the various strains, and then the agglutination of the strains of each group in the various antisera.

Chart 2.—Agglutination of group 1 strains in 9 antisera. Compare columns above with columns below for action of same antiserum on two strains of group 1.



Percentage agglutination of abortus 80 bacterium (group 1) in 9 antisera. The titer of each antiserum for its homologous strain is 100 per cent.

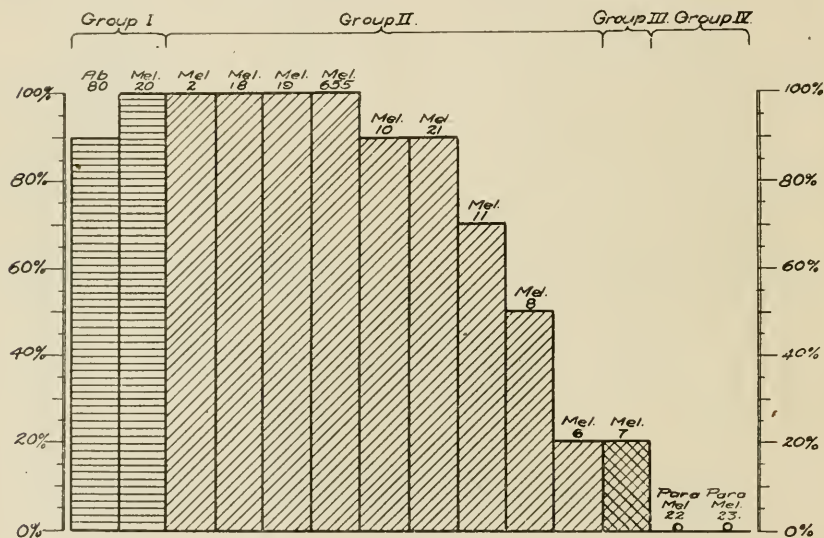


Percentage agglutination of Melitensis 20 bacterium (group 1) in 9 antisera. The titer of each antiserum for its homologous strain is 100 per cent.

Group 1 Antisera.—A potent antiserum of this group was characterized by definite gradations in the titer limits for the strains of the four groups. Group 1 strains agglutinated 100%. Group 2 averaged 60%, but in some of

the less potent antisera, such as those not exceeding 1:2,000, these strains agglutinated to the full titers, thus making no distinction in agglutinability for groups 1 and 2, a situation which will repeat itself later on. Group 3 strains did not react beyond 5%, and group 4 strains 1% or less. In those antisera in which group 2 strains reacted to the full titer, there was no proportional change in the titers for groups 3 and 4. Chart 1 represents a potent antiserum of group 1 showing the percentage agglutination for the experimental strains. In this antiserum the agglutination of group 2 averages 60%. Chart 9 represents the more common antiserum of group 1 in which the group 2 strains are flocculated to the titer limits. Strains 2 and 6 are somewhat irregular in most antisera, but the absorption tests assign them to group 2.

Chart 3.—Agglutinogenesis of group 2 antiserum (rabbit). Columns of like markings represent different strains of the same group.



Melitensis 19 antiserum (group 2). Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

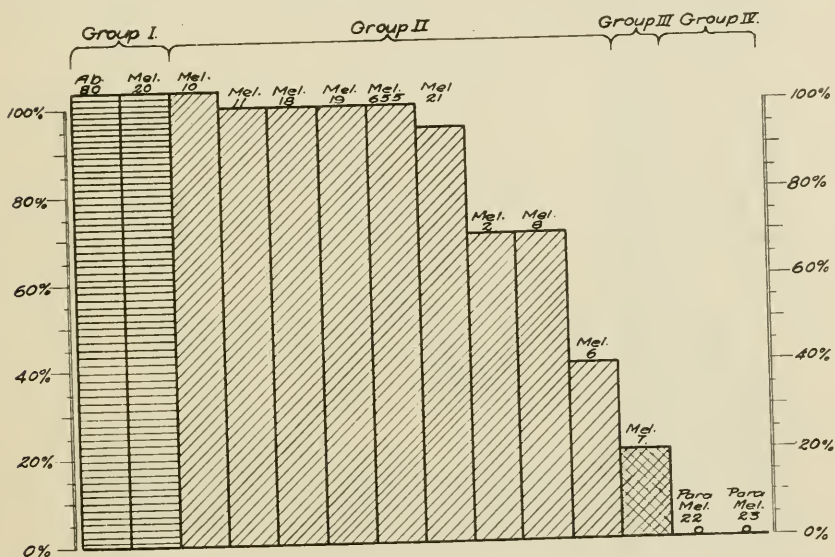
The 35 abortus strains showed a uniform range of 100% or thereabouts in all antisera of group 1 and apparently represent a uniform group. Chart 10 shows these *B. abortus* strains in a group 1 antiserum.

Group 1 Strains.—Whatever may be the inhibitory forces which prevent a group 1 antiserum from flocculating the strains of groups 3 and 4 to any marked degree, the same forces are in evidence when the strains of group 1 are agglutinated in the antisera of groups 3 and 4. About 40 *B. abortus* strains and *B. melitensis* 20 of group 1 could not react beyond 20% in group 3 antiserum. As group 4 (so-called paramelitensis) did not yield an antiserum in excess of 1:200, we limited our tests in this antiserum. Its agglutinins for group 1 fell below its titer. Group 2 antisera generally agglutinated group 1 strains to close to the titer limits. It will be seen that *B. melitensis* 2 anti-

serum was not very potent for group 1 strains, showing a similar irregularity to that of group 1 antiserum for melitensis 2 strain. Chart 2 shows the agglutination of 2 unselected strains of group 1 in 9 antisera.

Group 2 Antisera.—These antisera generally show no fundamental difference in the titers for strains of groups 1 and 2. They do not necessarily flocculate all strains to the titer limits, but the difference in degree of agglutinability is not sufficiently pronounced to establish a basis for separating the groups. Miss Evans' ⁶ y f strain—our *B. melitensis* 11—was of this group and from the reaction of its antiserum she concluded that "the agglutination reactions in *Bacterium melitensis* antiserum can distinguish *Bacterium abortus* from

Chart 4.—Agglutinogenesis of group 2 antiserum (monkey). Columns of like markings represent different strains of the same group.



Melitensis 655 antiserum (monkey), group 2. Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

Bacterium melitensis only when the agglutinating strength of the serum for both species is known." Other workers (Kennedy⁷) who have found a close relationship in the reactions for both species, were probably working with group 1 strains.

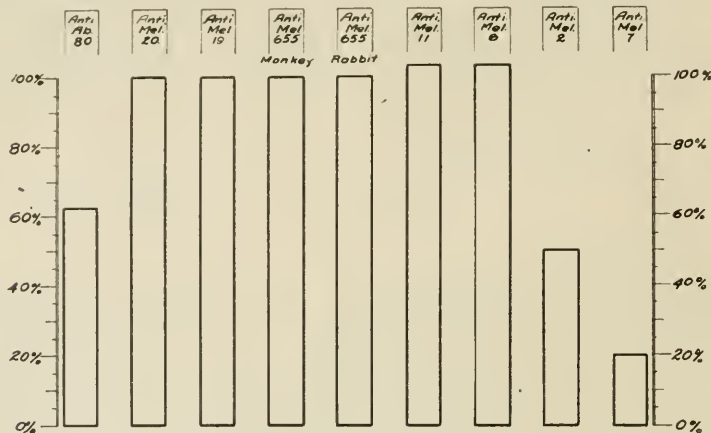
It can be stated that whenever there is reciprocal agglutination approximating the titer limits in *abortus* and *melitensis* antisera, the homologous strains must belong either to group 1 or to group 2. These groups cannot be separated from each other by absorption with a group 3 or a group 4 strain. They follow similar courses in such cases. They can be differentiated by reciprocal absorptions with their own group strains and antisera. The distinction is gen-

⁶ Jour. Infect. Dis., 1918, 22, p. 580.

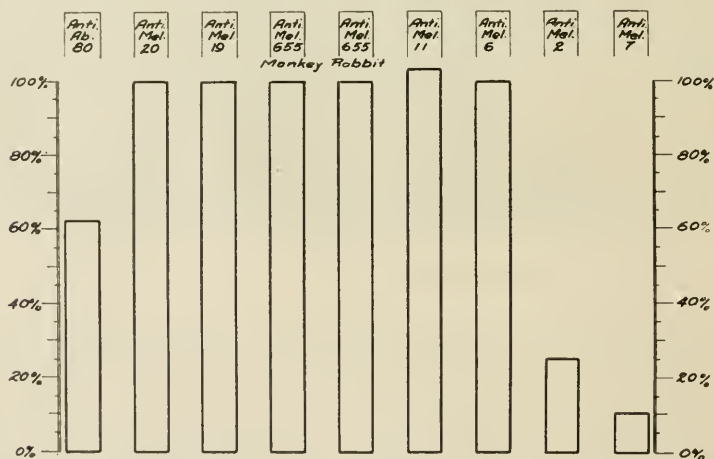
⁷ Jour. Roy. Army Med. Corps, 1914, 22, p. 9.

erally very delicate. All strains in this group are not equally agglutinable. Strains 2, 6 and 8 may fall considerably below the titer limits. Indeed the repeated irregularities of strains 2, 6 and possibly 8 (for which we had no

Chart 5.—Agglutination of group 2 strains in 9 antisera. Compare columns above with columns below for action of same antisera on two strains of group 2.



Percentage of agglutination of melitensis 18 bacterium (group 2) in 9 antisera. The titer of each antiserum for its homologous strain is 100 per cent.



Percentage of agglutination of melitensis 655 bacterium (group 2) in 9 antisera. The titer of each antiserum for its homologous strain is 100 per cent.

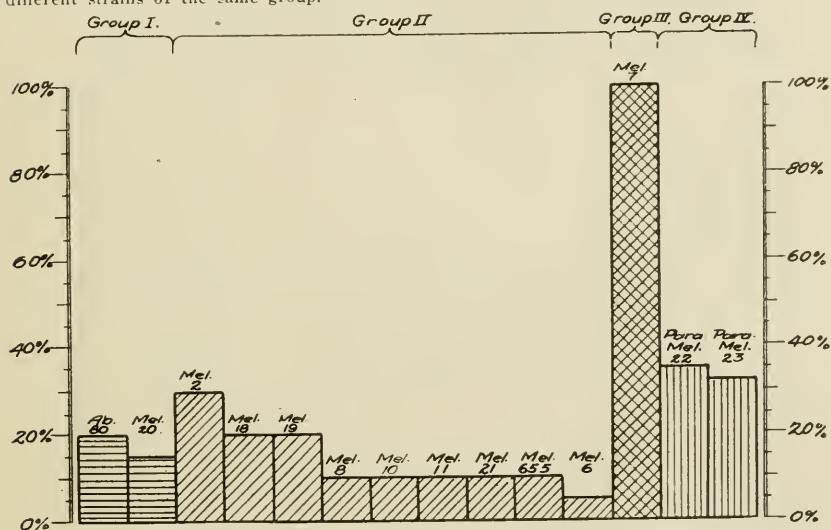
antiserum) suggest that their antigenic structure, if investigated, might justify placing them in a subdivision of group 2. Separately treated they would not distort the uniformity of the group nor distract the mind by constant reference to their irregularities. The titer of group 2 antiserum for group 3 strain

averages 20% except in antiserum 6 where it reacts to the titer limit. There are no demonstrable agglutinins in group 2 antisera for group 4 strains except in antisera 2 and 6 where there is a slight reaction.

Charts 3 and 4 show the titers of two antisera of this group for the experimental strains. Chart 3 is a rabbit antiserum with a titer of 1:2,000 and chart 4 a monkey antiserum with a titer of 1:20,000. It will be seen that the gradations are fairly uniform in the two antisera in spite of the striking differences in their titers and the fact that the immunization was made with 2 different strains of group 2.

Group 2 Strains.—As stated above, the strains of group 2 are agglutinated to about 60% or may be flocculated to 100% in group 1 antiserum. In group 3 antiserum they agglutinate from 10% to 20% of the full titer. Thus again we

Chart 6.—Agglutinogenesis of group 3 antiserum. Columns of like marking represent different strains of the same group.



Melitensis 7 antiserum (group 3). Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

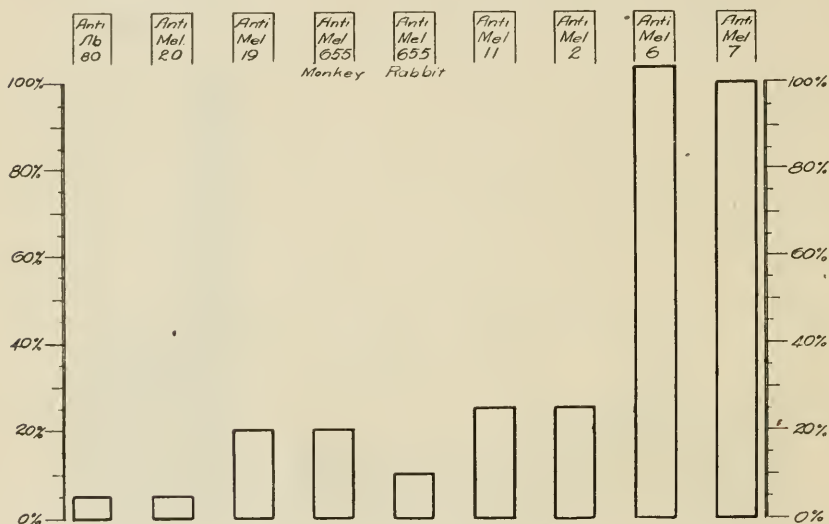
see the same inhibitory forces at work, confining the reaction of a group 2 antiserum on a group 3 strain and conversely the reaction of a group 3 antiserum on a group 2 strain to about 1/5 of their respective titers. In the weak antiserum of group 4 all strains of group 2 reacted to 1:200 which was the titer limit. Chart 5 shows the percentage agglutination of 2 strains of group 2 in 9 antisera.

Group 3 Antiserum.—The foregoing summaries have necessarily overlapped the reactions for groups 3 and 4. The agglutinins for group 3, which measured 100% for itself, are low for strains of groups 1 and 2, averaging about 1/5 of the titer limit. Melitensis 2 runs somewhat higher and melitensis 6 slightly lower than the other strains. Groups 1 and 2 cannot be separated from each other by agglutination in group 3 antiserum though they can be differentiated instantly from group 3 itself. On the other hand, group 3 is the only antiserum in which there is a fair agglutination for strains of group 4. These

readily react to at least 30% of the titer. As living suspensions they may agglutinate to 100% in this antiserum. Chart 6 shows the percentage agglutination of group 3 antiserum for the experimental strains, and chart 9 for the series of *B. abortus* strains which reacted from 10% to 20%.

Group 3 Strain.—This strain (our 7) probably the *M. pseudomelitensis* of Sergeant, Gillot and Lemaire,⁸ reacts to 5% in group 1 and to about 1/5 of the titer in group 2 antisera, and hence it is readily separated from them both. *Melitensis* 6 antiserum is a striking exception. It agglutinates group 3 to its titer limit. This is especially irregular because the antiserum of group 3 shows its minimum reaction on strain 6. Group 3 strain reacted to the full titer of 1:200 in the group 4 antiserum. Chart 7 shows the percentage agglutination of a group 3 strain in 9 antisera.

Chart 7.—Agglutination of group 3 bacterium in 9 antisera.



Percentage of agglutination of melitensis 7 bacterium (group 3) in 9 antisera. The titer of each antiserum for its homologous strain is 100 per cent.

Group 4 Antiserum and Strains.—This low titer antiserum did not show any differential reaction for the various groups except that group 1 ran slightly below the titer. The strains of this group are inagglutinable in most antisera. They show a slight reaction in group 1 antisera and in melitensis 2 and melitensis 6 antisera of group 2 in addition to their reaction in group 3 antiserum.

It will be seen from the subject matter presented above that we obtain striking gradations in agglutinability whether we consider the reaction of the antiserum on strains of the different groups or the reaction of strains in the antisera of different groups. Moreover, these gradations coincide with the groups established by the absorption tests. We may briefly summarize these gradations:

⁸ Ann. de l'Institut. Pasteur, 1908, 22, p. 209.

Group 1 antiserum does not generally differentiate between the strains of groups 1 and 2, though in an occasional potent antiserum it may do so. It agglutinates group 3 weakly and is agglutinated weakly by group 3. It shows a minimum reaction for group 4.

Chart 8.—Agglutination of group 4 strains in 9 antisera. Compare columns above with columns below for action of same antisera on two strains of group 4.



Percentage of agglutination of paramelitensis 22 bacterium (group 4) in 9 antisera. The titer of each antiserum for its homologous strain is 100 per cent.



Percentage of agglutination of paramelitensis 23 bacterium (group 4) in 9 antisera. The titer of each antiserum for its homologous strain is 100 per cent.

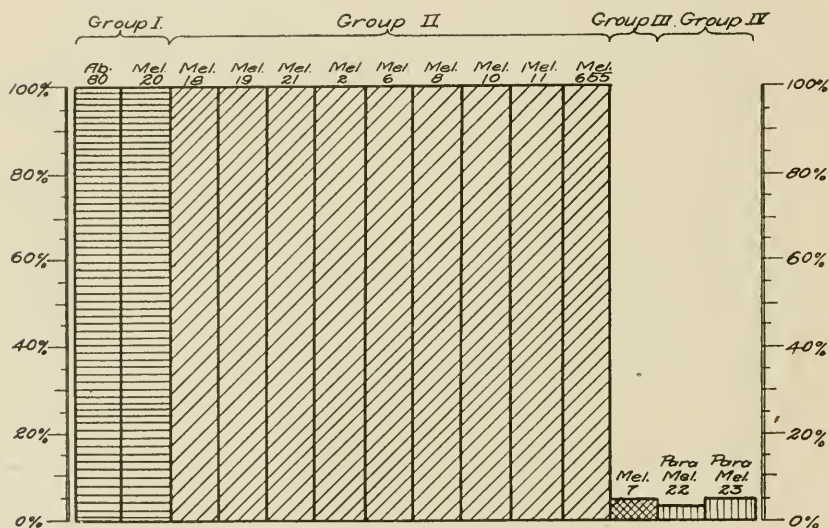
Group 2 antiserum shows no vitally specific differentiation between the strains of groups 1 and 2. It acts weakly on group 3 (except in antiserum 6) and is weakly acted on by group 3. It has no agglutinins for group 4 except in antisera 2 and 6.

Group 3 sharply differentiates itself from groups 1 and 2, and is equally sharply differentiated by them (except in antiserum 6). It agglutinates group 4 to at least 30% of its titer.

Group 4 is differentiated by its prevailing inagglutinability in most antisera and its inability to produce a potent antiserum in either rabbits or guinea-pigs.

The studies of Sergent and his co-workers,⁸ who reported finding "para" and "pseudo" melitensis strains, probably foreshadowed our grouping. Nègre and Raynaud⁵ identified one of Bruce's original organisms and gave it the name of paramelitensis. They also reported a race intermediate in agglutination between the "para" and the true melitensis.

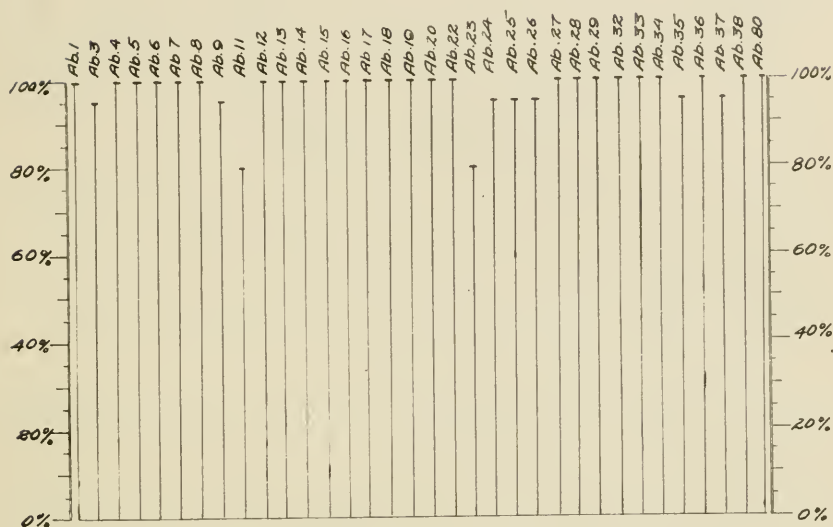
Chart 9.—Agglutinogenesis of group 1 antiserum. Columns of like marking represent different strains of the same group.



B. melitensis 20 antiserum (group 1). Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

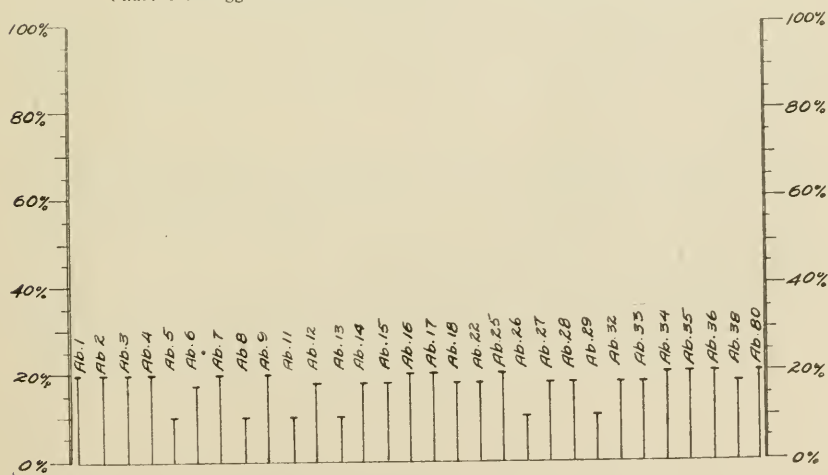
Through the courtesy of Dr. J. Traum, University of California, we obtained antisera from 2 cows and 3 hogs suffering from natural abortion disease. All these sera, except that of one cow, reacted to 1:200 with *B. abortus*. The excepted cow showed no reaction to *B. abortus* nor to *B. melitensis* strains. There was, however, in unheated serum a faint sedimentation with both group 4, the so-called paramelitensis strains. The other cow, on the contrary, yielded an antiserum which flocculated all strains of groups 1 and 2 to the titer limit, but showed no reaction for strains of groups 3 and 4. The 3 hog antisera flocculated *B. abortus* and showed a weak reaction for one or more *B. melitensis* strains, that is, 2 of the hogs gave a reaction for but one (not the same) *B. melitensis* strain, and the third for 6 melitensis strains. Group 3 was not agglutinated by any of the 5 antisera. It would seem that the animals, except one cow, were infected by a group 1 strain, the abortus group. From

Chart 10.—Agglutination of *B. abortus* strains in group 1 antiserum.



B. abortus 14 antiserum (group 1). Percentage of agglutination for *B. abortus* strains. The titer for the homologous strain is 100 per cent.

Chart 11.—Agglutination of *B. abortus* strains in group 3 antiserum.



B. melitensis 7 antiserum (group 3). Percentage of agglutination for *B. abortus* strain. The titer for the homologous strain is 100 per cent..

the above data we see that in immune serums naturally or artificially produced we obtain a reaction for both *B. abortus* and *B. melitensis* strains, which corroborates fully the observations of Evans⁶ and Kennedy⁷ with bovine serum and milk whey.

SUMMARY

Unless an antiserum is absorbed to extinction of the absorbing strain, the residual agglutinins cannot be classed as specific.

A series of absorption tests with formalized suspensions in *B. abortus* and *B. melitensis* antisera led to a fourfold grouping of 14 *B. abortus* and *B. melitensis* strains. Groups 1 and 4 were represented by 2 and group 3 by 1 strain, the majority fell in group 2. All *B. abortus* strains belonged serologically to group 1. Groups 1 and 2 are closely related. They are sharply defined from groups 3 and 4.

The grouping revealed these principles:

1. An antiserum cannot be exhausted by strains of another group. It is always exhausted by its homologous strain, and may be exhausted by other members of the same group.

2. A strain acts in a uniform manner (qualitatively) on all strains in another group under the same absorption conditions. This uniform action constitutes the basis for group affiliation.

3. Strains within the same group do not necessarily act in a uniform manner on one another when absorbed from the same antiserum. This constitutes the basis for individual differentiation.

In conforming to the main classification adopted by the Society of American Bacteriologists, we suggest that *B. abortus* and *B. melitensis* group be given generic rank in the *Bacteriaceae* family as the genus "*Brucella*."

A series of agglutination tests in *B. abortus* and *B. melitensis* antisera disclosed gradation in titer limits for the different strains and the gradations were constant for the same strains in the various antisera. It was found that the sets so formed correlated with the groups resulting from the absorption tests.

The serums of cows and hogs suffering from natural abortion disease may also react to both *B. abortus* and *B. melitensis* organisms.

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Preliminary Observations on the Pathogenicity
for Monkeys of the Bacillus
Abortus Bovinus

Wm. H. Meyer

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PRELIMINARY OBSERVATIONS ON THE PATHOGENICITY FOR MONKEYS OF THE BACILLUS ABORTUS BOVINUS*

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The importance of accurate determinations as to the pathogenicity of the *B. abortus bovinus* can hardly be overestimated, when one realizes that infectious abortion of cattle is present in practically all herds, and that the organism is invariably found from time to time in the milk of all infected animals.

In 1894, Theobald Smith called attention to a peculiar disease resembling tuberculosis occurring in guinea-pigs, following the inoculation of milk. It was not until 1911, however, that he, with Marshal Fabian as a co-worker, determined that these lesions were due to the *B. abortus bovinus*, and emphasized the desirability of discovering the possible rôle that this organism might play in human pathology. Since then the great prevalence of *B. abortus* in raw milk has been proven by numerous investigators, notably Schroeder and Cotton in this country and Zwick and Krage in Europe.

In 1916, Alice C. Evans published a most comprehensive piece of work on the bacteria of milk, using a special method of plating, whereby she determined that the *B. abortus* was present in about 25 per cent. of all samples studied. In 1915 and 1916, in an effort to learn the incidence of tubercle bacilli in certified milk, we injected the sediment of large quantities of milk into guinea-pigs, and found that inoculation experiments demonstrated the constant presence of *B. abortus* in the certified milk produced in the environs of San Francisco. Evans, in 1918, in a most illuminating piece of work, showed that the *B. abortus* and the *B. melitensis* were morphologically, serologically and biochemically practically indistinguishable. In 1919, following the injection of guinea-pigs with these organisms, we were able not only to confirm Evans' findings, but to prove that animals with abortion disease showed positive cutaneous hypersensitiveness,

*From the George Williams Hooper Foundation for Medical Research and the Department of Pediatrics of the University of California Medical School.

using either abortin or melitensin as an antigen, and, vice versa, those guinea-pigs with *B. melitensis* lesions gave equally strong skin tests, whether the abortin or melitensin was used. If one grants the usually accepted opinion as to the specificity in guinea-pigs of cutaneous hypersensitiveness, the close relationship of these organisms becomes more apparent.

Only by a series of absorption tests was it possible to differentiate the type of infection. The *B. melitensis* is the recognized cause of undulant fever in man. The disease occurs primarily in goats through whose milk human infection takes place with great ease. It is a striking fact that the clinical picture produced by the *B. melitensis* in goats is similar to that produced by the *B. abortus* in cattle, namely, infectious abortion, although both sets of animals may harbor their respective organisms, and having acquired a great degree of immunity, show few symptoms.

Even more striking than this, however, was the fact that in several sets of guinea-pigs, following the intratesticular injection of *B. melitensis*, the lesions, macroscopic and microscopic, occurring in the spleen and lymph nodes were indistinguishable from those produced by *B. abortus*.

In endeavoring to determine the pathogenicity of an organism for man, positive results in the lower laboratory animals are of little practical value. It becomes necessary, therefore, to gather data by endeavoring to infect monkeys. The literature records considerable work that has been done in infecting monkeys with *B. melitensis*. Not only by the subcutaneous and intravenous injection of the organism is it possible to get a definite infection, but by feeding a single sample of infected milk, the animals run characteristic temperatures and develop a disease which is very similar to that seen in man. Careful review of the work that has been done with the *B. abortus*, however, shows that only one investigator used monkeys in an effort to produce a characteristic disease. Marshal Fabyan infected two monkeys and was able to recover the organism twelve and thirteen weeks, respectively, after the inoculation. He does not state the method used in inoculating the animals and gives no additional observations as to the results.

In an effort to determine the pathogenicity of the *B. abortus bovinus* for monkeys, the following experiments were carried out:

RECORD OF EXPERIMENTS

MONKEY 1.—Feeding Experiment. April 3, 1919, fed 2 slants of *B. abortus* 152. Agglutination reactions April 9 and 21, and July 8 were negative. Died of intercurrent disease. Necropsy negative. Cultures negative.

MONKEY 2.—Intravenous Inoculation. April 3, 1919, injected one-fifth slant of *B. abortus* 80 (age of culture, 2½ years) into the left jugular vein. Good response by agglutinin production. May 24, chloroformed. Length of experiment fifty-one days. *B. abortus* isolated from the right and left kidney, spleen and liver. Agglutination tests: *Abortus* 80, 1:4,000 + and 1:1,000 + + +. *B. melitensis* 1, 2, 3, 4 and 5, 1:600 + + +, 1:1,000 + (Fig. 1).

MONKEY 3.—Feeding Experiment. Fed daily from July 2 to Aug. 24, 1919, between 150 and 50 c.c. of the milk of an infected goat.* From August 24 to Sept. 30, 1919, fed the same milk irregularly, depending on its production.

From October 4, to November 10, the monkey was fed daily one slant of *B. abortus* 80 and one slant of *B. abortus* 14 (age of culture, 10 years) on bread. He died November 12, of bronchopneumonia. (Duration of experiment, 133 days.)

B. abortus was recovered from the spleen. Serum of animal agglutinated *B. abortus* 80 in dilution of 1:600 + + + and *B. abortus* 14 in dilution of 1:200 + + +.

MONKEY 11.—Intravenous Inoculation. Aug. 6, 1919, injected one-quarter slant *B. abortus* 14 intravenously. Agglutination reactions August 21, fifteen days after inoculation, *B. abortus* 80 1:2,000 + + +, *B. melitensis* 1:800 + + and *B. melitensis* 1:600 + + +. Died November 12, of intercurrent disease. (Duration of experiment ninety-eight days.) Necropsy negative. Cultures contaminated. Agglutination reactions *B. abortus* 80, 1:40. *B. melitensis* all negative.

MONKEY 12.—Intravenous Inoculation. Aug. 14, 1919, injected intravenously one-quarter slant *B. abortus* 80. Monkey passage No. 2. August 18, animal died. (Duration of experiment four days.) *B. abortus* recovered from all of the organs; liver, spleen, lymph nodes, kidneys and bone marrow. Heart's blood sterile.

MONKEY 19.—Feeding Experiment. From Sept. 30, to Oct. 30, 1919, was fed goat's milk which was very rich in agglutinins, but very poor in organisms. Agglutination tests with the serum of this monkey were all negative. Died

* The history of the goat whose milk was used in this feeding experiment is as follows:

GOAT 1.—Infected June 2, 1919, by an injection of *B. abortus* 80 into the right udder. June 28, the milk contained from 80,000 to 100,000 *B. abortus* per c.c. July 8, the blood serum of the goat agglutinated *B. abortus* in dilutions of 1:1,000. The milk contained 20,000 *B. abortus* per c.c. July 17 the goat's serum agglutinated *B. abortus* 1:1,000 and the milk contained about 50 organisms per c.c. August 28, the cultures from the milk were sterile. September 1, tests of the blood serum of the goat were positive in dilutions of 1:600 + + + and the milk from the left udder agglutinated *B. abortus* 80 1:100 + + +. September 27, the left udder was reinfected with *B. abortus*. Milk from the right udder at this time gave positive agglutination tests, 1:100 + +. October 22, there was no longer any secretion of milk from the udder.

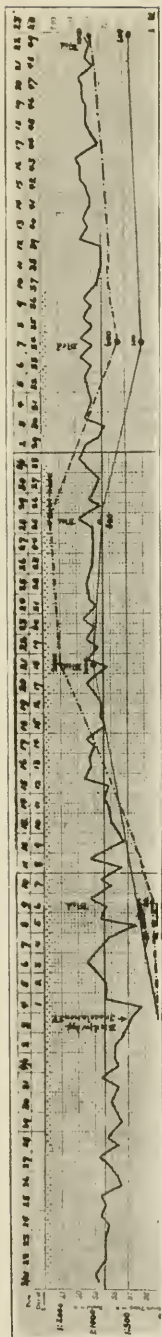


Figure 1

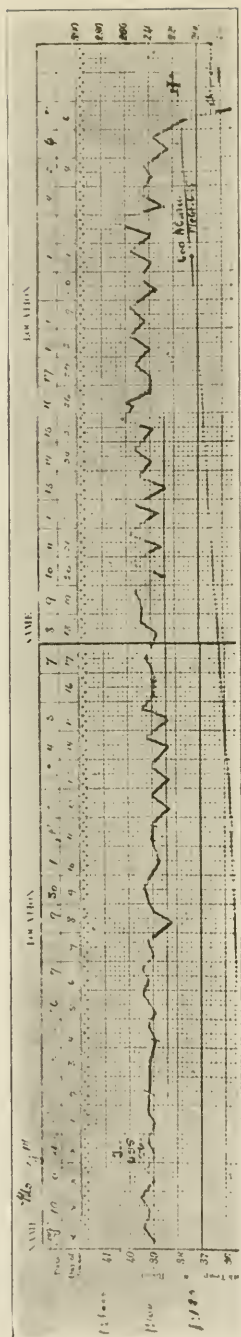


Figure 2

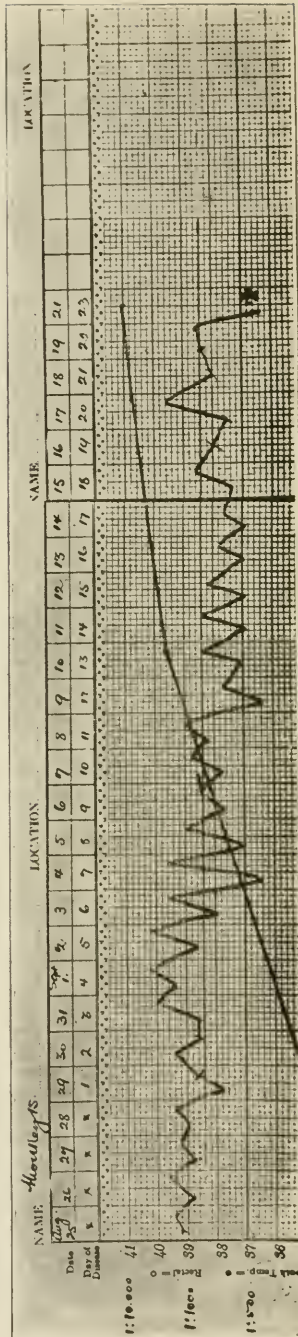


Figure 3

November 8, from bronchopneumonia. (Duration of experiment thirty-nine days.) Cultures from all organs were negative. Agglutination tests negative.

MONKEY 13.—Feeding Experiment. From Aug. 5, to Aug. 24, 1919, fed one slant each of *B. abortus* 80 and 14 on bread or carrots. Aug. 27, 1919, animal was chloroformed. (Duration of experiment twenty-two days.) Necropsy showed a very emaciated animal. Cultures were positive from the mesenteric lymph nodes and spleen. Agglutination reactions were positive: *B. abortus* 80, 1:100+, *B. abortus* 14, 1:100+, and *B. melitensis* 655, 1:100+.

MONKEYS 34 AND 35.—Feeding Experiment. Fed one slant of *B. abortus* 80 and 14. Monkey 34 has shown positive agglutination tests 1:100 on several occasions. Experiment still running.

MONKEYS 36 AND 37.—Feeding Experiment. Have been fed daily pasteurized milk to which has been added *B. abortus* 80 and 14. Agglutination reactions 1:200+++. Experiment still running. Up to the present time have developed no positive agglutinins in the blood.

MONKEYS 38 AND 39.—Feeding Experiment. Have been fed daily pasteurized milk to which has been added one one-hundredth slant *B. abortus* 80. No reaction in the blood. The animals developed a diarrhea, the cause of which up to the present time has not been explained.

MONKEY 40.—Feeding Experiment. From March 30, 1920, for fifty-two days, fed daily the milk of a goat that had been infected with a virulent *B. abortus* recovered from an infected hog. April 23, after twenty-four days of feeding, the milk from the right udder of the goat contained 1,500 organisms per c.c., and from the left udder 250,000 organisms per c.c. May 10, forty days after the beginning of the experiment the milk from the right udder contained 30,000 organisms per c.c., and from the left udder 180,000 organisms per c.c. April 23, after twenty-four days of feeding, agglutination reactions were negative. They were also negative May 7, thirty-seven days after beginning of experiment. May 19, forty-nine days after beginning of experiment, agglutination reactions were positive in dilutions of 1:200. The animal was sacrificed on the fifty-second day. Had lost 300 gm. in weight. The lymph nodes were very much enlarged, a spleen tumor was present and *B. abortus* was recovered in enormous numbers from the spleen, lymph nodes, bone marrow and kidneys. Blood cultures from the heart were strongly positive.

MONKEY 42.—Feeding Experiment. Fed daily milk of a goat which had been infected with hog abortus. Agglutination reactions were positive in the third week. Experiment still running.

MONKEYS 43 AND 44.—Feeding Experiment. Fed one slant of *B. abortus* hog daily. Agglutination reactions with the serum of Monkey 44 were positive in third week. Experiment still running.

In order to prove the melitensis character of the strain of *B. melitensis* which was pathogenic for guinea-pigs, following intratesticular injection, the following two experiments were carried out.

MONKEY 14.—Weight, 2,830 gm. Subcutaneous inoculation. Aug. 21, 1919, was inoculated subcutaneously with strain 655 *B. melitensis*. Nearly dead September 27. Chloroformed. (Duration of experiment thirty-seven days.) Weight, 2,250 gm. Cultures: spleen, 100 colonies; liver, one colony; right kidney, twenty colonies (Fig. 2).

MONKEY 15.—Weight, 2,010 gm. Intravenous inoculation. Aug. 28, 1919, injected *B. melitensis* 655 into right saphenous vein. September 21, comatose. Chloroformed. (Duration of experiment thirty-one days.) Cultures positive from the bone marrow, right and left lung, spleen, urine and liver, *B. melitensis* 655. Agglutination reactions *B. abortus* 80, 1:20,000 + + +, *B. melitensis*, 1:10,000 + + +, and *B. melitensis* 655, 1:10,000 + + + (Fig 3).

CONCLUSIONS

It is not justifiable to draw absolute conclusions from a limited number of experiments, but a careful survey of the data reveals some very interesting probabilities.

1. Following the intravenous injection of *B. abortus bovinus*, agglutinins develop fairly rapidly in the blood of monkeys, and on postmortem it is possible to recover the organisms from the spleen, lymph nodes, liver, kidneys and less frequently from the bone marrow.

2. By feeding agar slants of *B. abortus* on bread or carrots in large and repeated doses, it is possible to get strongly positive agglutination reactions and to recover the organisms from all the viscera at necropsy.

3. By feeding repeatedly the milk of a goat, that has been infected by the injection into the udder of a very virulent strain of *B. abortus* recovered from a hog, it is likewise not only possible to recover the organism from all the viscera, but to determine its presence in the circulating blood.

4. It seems, therefore, justifiable to assume that the *B. abortus bovinus* is pathogenic for monkeys if a virulent culture is fed in large quantities, showing that it has the capacity to penetrate what is apparently a normal mucous membrane of the intestinal tract.

5. In view of the fact that the strain of *B. melitensis*, which produced characteristic lesions in guinea-pigs resembling those of *B. abortus*, when injected into monkeys gave rise in temperature, positive agglutination reactions, and was recovered postmortem in pure cultures from the viscera of the animal, it seems logical to state that this is a true *B. melitensis*.

Irregular Typhoid Strains and the Infections
Caused by Them

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IRREGULAR TYPHOID STRAINS AND THE INFECTIONS CAUSED BY THEM

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In a review recently written by V. C. Vaughan¹ on a report published from the Public Health Laboratories of Cairo, Egypt, entitled "The Bacteriologic Examination of Suspected Typhoids," the following statement is made:

"It is possible and indeed highly probable that so far as vaccination has failed it is due to the disease being caused by other members of the typhoid group, which in all probability is much larger than we now appreciate. A second most interesting point brought out in this valuable report is that of the introduction of a disease into a country where it has not hitherto prevailed and the possibility of the newly introduced organisms supplanting kindred organisms already native to the country. There are many reasons for believing that in the various camps in this country different organisms became predominant and the dominating organisms changed from time to time with new importations. It is possible that the great differences in the death rates in the various camps in this country may have been due to this or similar causes."

The observations reported in this paper lend some support to the suggestion mentioned, namely, that the failures of antityphoid-paratyphoid vaccination may be the result of other members of the typhoid group or, as it appears in our case, to be variants of typical *B. typhosus*. Minor variations among strains of the same species of bacteria are not uncommon, but in rare instances it has been possible to detect some of the factors that induce the production of such variants. With the creation of a large stratum of population highly protected by vaccination against typhoid and paratyphoid fever, the appearance of isolated cases or of small group epidemics in the supposedly immune must suggest an explanation similar to the one offered in the review. Unfortunately, the analysis of the various strains of *B. typhosus* isolated from vaccinated typhoid fever cases are few, the available data are unreliable and mostly obtained by incomplete biochemical and

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¹ Jour. Lab. & Clin. Med. 1919, 4, p. 645.

serologic methods. In fact, the recent findings of F. Mock² in 45 positive typhoid and paratyphoid fever cases at Mesves Hospital in France, representing cultures from vaccinated soldiers, emphasize this statement in every respect. It is not surprising to find in his paper, dealing with variants and irregular strains of the typhoid and paratyphoid group of bacteria, the conclusion that "these atypical paratyphoid organisms probably are involution forms of the true typhoid and paratyphoid bacteria." Some of his strains changed their cultural while others exhibited irregularities of their agglutination characteristics. It is therefore not unlikely that a more careful analysis of these strains would have forced a modification of the sweeping conclusions and would have led to a consideration of the epidemiologic importance of such atypical strains.

A review of the literature indicates that irregular typhoid and paratyphoid strains have been repeatedly reported.³ Recent publications also call attention to the existence of nongas-producing paratyphoid and *B. enteritidis* strains, which may be readily mistaken for true *B. typhosus*, when only abbreviated methods for identification are employed.⁴ In this connection we recall the conclusions of Tenbroeck⁵ in his paper on a nongas-producing hog cholera bacillus in which the statement is made that his strain resembles in many respects *B. typhosus*, and it may be that some of the so-called typhoid cultures that are not agglutinated by antityphoid serum are nongas-producing paratyphoids. A detailed consideration of the other publications will be given in connection with the discussion of our own results.

Even since 1913, when one of us (K. F. M.)⁶ analyzed rather superficially an irregular strain of *B. typhosus*, which had been isolated from a vaccinated army officer, we have studied several hundred typhoid and paratyphoid cultures isolated from man or after prolonged sojourn in the tissues of laboratory animals. Only recently two irreg-

² Jour. Lab. & Clin. Med. 1919, 5, p. 54.

³ See the publications of: LeCount and Kirby: Trans. Chicago Path. Soc., 1903-1904, 6, p. 209; Faroy: Compt. rend. Soc. de biol., 1908, 64, p. 1093; Lafforgue, *ibid.*, 1908, 65, p. 109; Marotte: Progrès méd., 1909, No. 28, p. 358; Babes and Feodorascu: Compt. rend. Soc. de biol., 1909, 66, p. 787; Fromme: Centralbl. f. Bakteriöl., 1911, 58, p. 445; Goebel: *ibid.*, 1914-15, 75, p. 376; Niole, Raphaël et Debains: An. de l'Inst. Pasteur, 1917, 31, pp. 373, 388 and 403; Raynaud and Nègre: Compt. rend. Soc. de biol., 1912, 72, p. 534; MacAdam: Jour. Roy. Army Med. Corps, 1919, 33, p. 140.

⁴ Messerschmidt: Centralbl. f. Bakteriöl., 1912, 66, p. 35; Oette: *ibid.*, 1913, 68, p. 1; Wagner: *Ibid.*, 1913, 71, p. 25; Ohno: *ibid.*, 1915, 75, p. 288; and Morse and Tyron: Boston Med. & Surg. Jour., 1917, 177, pp. 173, 216 and 255; Broughton-Alcock: Lancet, 1919, 2, p. 1023.

⁵ Jour. Exper. Med. 1916, 24, p. 213.

⁶ See footnote 2 in Riesman: Jour. Am. Med. Assn., 1913, 61, p. 2205.

ular typhoid strains isolated from two vaccinated laboratory workers attracted our attention. The epidemiologic circumstances leading to the infection and the interpretation which our observations suggest justify in our opinion a detailed account of the clinical and bacteriologic observations. The evidence to be presented also indicates that one of the irregular strains reverted to its typical ancestor by passing through an aged nonimmunized man. The history of the infections and the bacterial findings are:

CASE 1.—A janitor in our animal house, aged 59, complained of chilly sensations, weakness and severe frontal headache on April 1, 1919. He had a temperature of 38.8 C. and was therefore admitted to the hospital. There was nothing noteworthy in his family history. About March 10, 1919, he had felt chilly and feverish for two or three evenings in succession, but in the mornings he was always able to attend to his duties. However, during the day he perspired freely and was more readily fatigued in carrying his usual load of feed and distilled water, etc., upstairs. On account of the severe illness and the death of his wife he had very little rest during the months of January and February. In July, 1917, he was given three injections of fresh army T. A. B. vaccine; each injection was followed by a moderate local reaction. He has had for the last two years intimate contact with rabbits, guinea-pigs, cats, dogs and goats that discharged living typhoid bacilli. The two months preceding his illness about 30 to 40 rabbits, either renal or gall-bladder typhoid bacilli carriers, and numerous guinea-pigs infected spontaneously with rodent paratyphoid B. bacilli and B. enteritidis were kept in the section of the animal house under his supervision. The same caretaker handles also the cremation of these animals, which are always carefully wrapped by the experimenter in heavy paper. He never took any meals in the animal house and used water, soap and cresol solutions before returning to his home. However, in his spare moments he smoked cigarets, which he rolled himself.

Physical Examination.—The patient was well nourished and well developed; aside from his flushed cheeks he did not look very ill. There were no rose spots and no glandular enlargements. Liver and spleen were not felt. Both lungs showed normal dullness and breath sounds. The urine showed no albumin, sugar or sediment; the diazo reaction was negative. The blood count was 4,300,000 and 7,200 with 65% neutrophils and 24% lymphocytes. Temperature was 38.6 degrees, pulse 90, and respiration 26.

From the blood cultures (5 cc of blood in 200 cc of glucose broth and 2 cc in 10 cc glycerin peptone ox bile) (taken in the afternoon of April 1, 1919) a gram-negative typhoid-like organism grew in 18 hours. The agglutination test made on the same day was:

B. typhosus formalinized antigen, 1:10, + + +.

B. typhosus living polyvalent, 1:40, + +.

B. typhosus paratyphosus A, formalinized, 0.

B. typhosus paratyphosus B, formalinized, 0.

On the third day of the patient's stay in the hospital, during which time the temperature did not rise over 38.2 C., it fell to 37 C. and remained normal. On April 5 and 9 he received intravenously 20,000,000 each of a polyvalent mixture of several strains of B. typhosus in addition to his own organisms.

The provocative injections produced a very slight hyperleukocytosis, blood cultures taken immediately after the clinical reaction remained sterile. Stool and urine cultures taken daily for 14 days failed to demonstrate organisms that could be identified with those isolated from the blood. The patient left the hospital on April 9 and returned to his work perfectly well on the 20th. On April 21 and 27 he received subcutaneously one billion each of the heatkilled, tricresolized bacteria, that is, the organism isolated from his blood. The agglutinations before and after the injections were as follows:

AGGLUTINATIONS BEFORE AND AFTER INJECTIONS OF BACTERIA

Date	Bacillus Isolated from Blood, Living	B. typhosus Formalinized	B. paratyphosus A Formalinized	B. paratyphosus B Formalinized
April 5	0	1:20 ++	0	0
April 8	0	1:20 ++	0	0
April 21	1:10 ++	1:20 +++, 1:40 ++	0	0
April 27	1:80 ++	1:40 +++	0	0
May 2	1:80 ++	1:40 +++	0	0

As several independent workers, by the use of the ordinary procedures, classified the bacillus isolated from the blood stream of the caretaker as a typhoid bacillus, the malady was also clinically diagnosed as a mild abortive form of typhoid fever in an aged and vaccinated man. Subsequent observations on the original milk and carbohydrate tubes and repeated agglutination tests threw doubt on the original identification and a more detailed study was contemplated as soon as other duties in the laboratory permitted. Such an inquiry became a necessity when another laboratory worker contracted an infection which was clinically diagnosed as typhoid fever and an organism similar to the one found in case 1 was isolated from the urine. The history of the second case is:

CASE 2.—A woman, aged 26, graduate student in bacteriology in this laboratory, complained of headache, general malaise, abdominal pains and remained absent from her work on June 2, 1919. For the last two months she had assisted in making thousands of agglutination tests of suspected typhoid colonies; in particular she had made several agglutination and fermentation tests with the bacillus isolated from case 1. In the course of these tests she examined also various plates that contained organisms of the *B. enteritidis* and *B. paratyphosus* *B. rodent* group. Her technic was clean and careful, and she always disinfected her hands thoroughly before leaving for meals. In July, 1918, she was vaccinated with T. A. B. Navy vaccine at Mare Island; each injection produced a moderately severe reaction. In October, 1919, she had a severe attack of pandemic influenza.

From June 2 until June 15, neither of us saw the patient, who lived out of the city, but a tentative diagnosis of abortive typhoid was suspected by the physician. To confirm this diagnosis we offered our services and con-

ducted repeatedly laboratory tests which are summarized in table 1. About June 20, the patient having been afebrile was permitted to leave the bed. She suffered a relapse on June 22 and was after that attended regularly by a physician and a nurse. The temperature chart available shows two typical enteric fever relapse curves, one extending from June 28 to July 12 and the other from July 14 to July 25. The only clinical data available state that the course was severe, accompanied in the last relapse by delirium. No rose spots were noticed, the spleen was never distinctly palpable but the pulse and blood count were characteristic for typhoid fever. Beginning June 28 she received a high calory carbohydrate diet. Her recovery was uneventful and complete. Our laboratory findings, which have a bearing on the problem to be discussed, are for the sake of clearness presented in tabulated form.

TABLE 1
LABORATORY FINDINGS IN CASE 2

Date	Leukocytes	Agglutination		Blood Cultures	Urine Cultures
		B. Typhosus	B. Para A, B, Dysenteriae, B. Melitensis		
June 15	7,300	1:20	0	Sterile	Negative
June 25	9,300	1:100	0	—	Negative
July 4	—	1:640	0	—	Positive, 6 colonies
Aug. 27	—	1:1000+	1:10-1:100	—	—

The facts stated, in conjunction with a consideration of the bacteriologic findings, are of considerable interest. A laboratory worker vaccinated against typhoid and paratyphoid developed a clinically typical typhoid fever infection. Agglutination and blood cultures failed to support this diagnosis until a bacillus identical in every respect to the one found in case 1 was isolated from the urine in the fifth week of the disease. Blood cultures were taken only in the second week, when the temperature was declining and the stool cultures were undoubtedly negative on account of the high calory carbohydrate diet, which had been given to the patient since June 10. The patient apparently was shedding organisms in the first two to three weeks' period of her illness as was indirectly demonstrated by the occurrence of another typical case of typhoid fever in her household. The history of this patient is:

CASE 3.—The father of patient in case 2, aged 70, unvaccinated, complained of malaise and headache on July 24. From that date until August 9 his temperature rose to 102 and 104 F., but he was not sufficiently ill to follow the advice of his physician which was to remain in bed. On August 9 agglutination was found positive by a board of health laboratory, and he was subsequently kept in bed. From the few data available it is evident that his typhoid infection was typical, very severe and ended fatally on Sept. 8, 1919. The clinical diagnosis was well supported by a few examinations we were able to conduct on the patient on Aug. 25, 26, and 29, 1919.

FINDINGS IN CASE 3

Date	Agglutination		Stool Culture	Urine Culture
	B. typhosus Formalinized	Case 1 Bacillus, Living		
Aug. 25	∞ typical B. typhosus	
Aug. 26	∞ B. typhosus
Aug. 27	300 million B. typhosus per c c of urine 4 hours after collection
Aug. 29	1:400 +++; 1:800 ++	1:600 +++; 1:800 ++	Almost pure B. typhosus	90 million B. typhosus per c c of urine 9 hours after collection

As Sch. never entered the room of the patient and the most scrupulous precautions in sterilizing all secretions had been taken by the nurse who attended case 2 since June 28, it was for a considerable period impossible to connect his infection with the one of his daughter, discussed in case 2. Sch. had remained in his home and our most searching epidemiologic inquiries failed to find an outside source where he could have contracted the disease. On further detailed analysis of the circumstances leading to the illness of her father, the daughter remembered that on or about June 20 when very ill she prepared unknown to her mother who attended her a specimen of her own stool and instead of sterilizing the applicator, threw it in the toilet. On July 12 a plumber was called to clean the clogged siphon. Sch. assisted him, removed and handled the applicator and commented to his wife and the nurse on the negligence of the person, who threw the piece of wood in the lavatory. Twelve days later on July 24 he noticed the initial symptoms of his typhoid infection.

The occurrence of this indirect contact case would in itself, as from an epidemiologic point of view, be of little value, but in correlation with the bacteriologic findings, the history can be appreciated and analyzed more carefully.

BACTERIOLOGIC IDENTIFICATION OF THE ORGANISMS ISOLATED FROM CASES 1, 2 AND 3

As stated in the histories, the bacillus isolated from the blood stream of case 1 behaved irregularly when tested by more detailed carbohydrate and serologic tests; the organism found in one urine sample of case 2 corresponded with the organism of case 1, with the exception of a marked hyperagglutinability in the first 16 transplants on digest or veal agar. The organism isolated from case 3 was easily identified as a typical typhoid bacillus. In the course of the epidemiologic analyses of the recorded findings it became necessary to compare the isolated organisms with the various typhoid strains to which the laboratory workers were suspected of having previously been exposed. It was, however, impossible to demonstrate conclusively the strain or strains of B. typhosus, which by passing through the body of case 1 had

become altered to an irregular typhoid bacillus, nor did we collect observations which could prove the infections were the result of an irregular *B. enteritidis*. Before discussing the various tests employed for the identification we state briefly the technical procedures used.

Blood cultures were made with from 5 to 10 cc of blood in glucose veal infusion broth (P_H 7.0) and peptone-glycerol-ox bile. Stool and urine specimens were plated on brilliant green-eosin-peptic digest agar. Urine samples were also enriched with an equal amount of peptic digest broth.

The isolated colonies of gram-negative nonlactose fermenting organisms were purified by repeated successive plating on peptic digest or veal agar. The three strains studied in this paper were also isolated by Burri's method as one cell cultures. The progenies of three cells of each strain were studied in peptone water-potassium phosphate-sodium chlorid-carbohydrate solutions with Andrade's or China blue rosolic acid indicator, Witte's peptone solution, bromcresol purple milk, neutral red, orcein and malachite green solution in 0.5% meat extract agar, and rhamnose-veal agar. The P_H reaction of all mediums used was adjusted to 7.0-7.2. It will be shown in another paper that strain "I 75" of case 1 when first isolated was alkaline tolerant; the growth curve showed a marked plateau extending from P_H 6.8 to 7.8. All tests reported have been repeated at least three times and the findings with a few minor exceptions to be discussed in detail remained constant. The parasitic strains differed in no way biochemically from the saprophytic ones; however, this statement cannot be applied to some of the serologic findings on the saprophytized offsprings of the two strains "I 75" and "Chr. 76." It appears advisable for the sake of clearness to discuss the various characteristics under separate headings.

Morphology.—The three strains "I 75," "Chr. 76" and No. 49 are morphologically indistinguishable from the typhoid type strain "Rawlings"; they are gram-negative and actively motile. They show differences in size during their growth on mediums identical with those described by Clark and Ruehl;⁷ on very alkaline mediums filamentous rods are frequently noted.

Surface Colonies.—On dye mediums or on plain agar the parasitic strain of "I 75" and "Chr. 76" produced vine leaf shaped granular colonies. As a rule the colonies were always somewhat larger and the characteristic growth permitted recognition of the irregular strains in a mixture with a typical *B. typhosus*. The inside structure shows a rather fine striated network of furrows, which are readily visible with the naked eye. Indeed the colonies correspond in many respects with those recently described by v. Lingelsheim and Sachs-Mücke⁸ as so-called Q-strains. Recent tests with the more saprophytic strains produced irregular grayish or slightly yellowish lobulated colonies, which developed raised centers and some isolated colonies may occasionally show indications of slimy edges. These changes occur only when the plates after incubation for 18 hours at 37 C. are kept at room temperature and again the mucous appearance of the edges is only slight in comparison with those constantly noted on typical paratyphoid *B.* strains. On gelatin plates the typical leaf-like appearance of the colonies is more pronounced than on the agar plates, the medium is never liquefied.

⁷ Jour. Bacteriol., 1919, 4, p. 615.

⁸ Centralbl. f. Bakteriol., 1913, 68, pp. 577 and 582.

Lead Acetate Reaction.—The medium prepared according to Jordan and Victorson⁹ is slowly reduced without the production of gas; the hydrogensulfide reaction is identical with the one noted for typical typhoid strains tested simultaneously.

Carbohydrate Reactions.—Strain "I 75" and "Chr. 76" ferment without gas production the following carbohydrates: glucose, levulose, galactose, mannose, mannite, maltose, xylose, dextrin, arabinose (3 times of 5 tested), dulcitol and rhamnose. Strain "49" failed to ferment arabinose, dulcitol and rhamnose in the observation period of 30 days. It is generally stated in textbooks and emphasized by Winslow, Kligler and Rothberg¹⁰ in their studies on the classification of the colon typhoid group, that the type strain "Rawlings" does not attack arabinose, dulcitol or rhamnose. Recent studies by Teague and Morishima¹¹ confirming previous observations made by Penfold,¹² Wagner,³ Dittborn¹³ and others indicate that at least 6% of their typhoid cultures showed acid production in arabinose and from 14 to 37% in dulcitol broth, when the period of observation was extended to 30 days. Of the 14 typical typhoid strains, which are under suspicion of containing the strain responsible for the infection in case 1 and used by us for comparison, 2 or 14% fermented repeatedly arabinose on the 7th or 14th day and 6 or 48% acted on dulcitol in the one test, in which all strains were tested simultaneously. Repeated tests of our strain "I 75" and "Chr. 76" in arabinose-peptone-indicator solution confirmed the observations of Teague and Morishima that the acid production in this carbohydrate is irregular. In an early series with the parasitic strains acid production was noted in from 4 to 6 days; in another series with the saprophytic strains the reaction was delayed for 15 and even 24 days. On the other hand, the fermentation of dulcitol was fairly regular; as a rule, acid was formed in from 2 to 4 days; in one series of tests a delay of 8 days was recorded. In some tests with dulcitol the indicator was slightly reduced.

On endoplates prepared with arabinose instead of lactose, strain "I 75" and "Chr. 76" produced inside of the large isolated colonies in from 5-7 days one or several bud-like daughter colonies. Transplants from the papillae fermented arabinose in 24 hours. These observations on fuchsin-arabinose-agar are in many respects similar to those described by one of us (K. F. M.) for the paracolon bacilli isolated from calfscur's.¹⁴

On dulcitol-endoplates also red papillae are produced about the 8th to 10th day, but transplants from these behaved irregularly; an observation which we found confirmed by the recent publication of Teague and Morishima.

The fermentation of rhamnose or isodulcitol is regularly noted in any liquid medium chosen. Sometimes 3-5 days elapse before distinct acid reaction is shown by the indicator; in some series the acid production was confined to the flocculent growth sediment of the tubes and only 3-4 days later the acidity diffused throughout the liquid. According to Krumwiede, Kohn and Valentine¹⁵ and Winslow, Kligler and Rothberg,¹⁰ who have recently tested a series of typhoid strains, it is generally believed that the *B. typhosus* does not ferment this particular carbohydrate and the bacillus can therefore readily be differ-

⁹ Jour. Infect. Dis., 1917, 21, p. 554.

¹⁰ Jour. Bacteriol., 1919, 4, p. 472.

¹¹ Jour. Infect. Dis., 1920, 26, p. 52.

¹² Jour. Hyg., 1912, 12, p. 195.

¹³ Centralbl. f. Bakteriöl., 1912-13, 67, p. 497.

¹⁴ Jour. Infect. Dis., 1916, 19, p. 700.

¹⁵ Jour. Med. Research, 1918, 38, p. 89.

entiated from the members of the paratyphoid group. Penfold,¹⁶ on the other hand, states that "growth of *B. typhosus* on isodulcitol broth frequently does not produce acidity though it may do so as early as one week." Thus it is quite evident that this pentose in a liquid substratum is not of much value for distinguishing irregular strains of *B. typhosus* from nongas-producing paratyphoid strains.

Rhamnose-Agar Papillae Reaction.—In this connection it should be recalled that R. Müller¹⁷ and later Penfold,¹⁶ Saisawa,¹⁸ Wagner,³ Teague and Morishima¹¹ consider the development of daughter colonies on rhamnose agar a specific reaction for typhoid bacilli. In a series of tests with a small amount of rhamnose available we were able to confirm this specificity. Strain 49 and 4 other typhoid strains, representatives of the three groups of Hooker's serologic classification produced immumerable dense papillae in from 48 to 72 hours. Strain "I 75" and "Chr. 76" in spite of vigorous growth developed only small daughter colonies about the 10th or 12th day of incubation; they rarely reached the size of those noted with the typical typhoid strains and always remained translucent. Strain "I 75" and "Chr. 76" evidently differ in the rhamnose-papillae reaction from the typical typhoids which in part explains their ability to ferment this carbohydrate by acid production. The freshly isolated, as well as the saprophytic, strains behave in an identical manner. Thus far no reversion to the true type has been observed.

Raffinose-Agar Papillae Reaction.—Neither of the three strains concerned in this publication produced papillae on raffinose agar. The animal strains of *B. paratyphosus* B and *B. enteritidis* to which the workers had been exposed, all showed centrally located daughter colonies.

Little need be said with regard to the fermentation of xylose. We fully agree with Teague and Morishima that the so-called xylose nonfermenters are in reality slow fermenters. Strain "I 75" and strain "Chr. 76" were rapid xylose fermenters and maintained this property when repeatedly tested during the last 8 months.

Bromcresol-Purple Milk.—Both strains "I 75" and "Chr. 76" are characterized by rapid alkaline production in milk. The initial slight acidity is changed on the 3rd to the 5th day to a decided alkalinity which progressively increases and leads to saponification of the milk fat on the 15th to 30th day. The rapidity with which the reaction changes from a P_{H^+} 6.6 to P_{H^+} 9.0 in this medium has somewhat slowed down in these cultures kept for 6 months on plain peptic digest agar. The two strains as second and third generations isolated from the human body produced a deep purple reaction (P_{H^+} 8.6) inside of 5 days. Quite recently tested, at least 10 to 15 days elapsed until the same degree of alkalinity under the same conditions was noted. We have made similar observations on several strains of *B. sanguinarum* kept on agar for nearly 4 years; originally rapid they have gradually changed to slow alkali producers. It was noted in a series of tests with strain 49 that often at the end of 30 days' incubation about 50% of the inoculated milk tubes gave decided alkaline reactions. For example, transplants made from 12 isolated colonies on plain agar into milk tubes of the same lot, produced after varying intervals the following reactions:

¹⁶ Brit. Med. Jour., 1910, 2, p. 1672.

¹⁷ Centralbl. f. Bakteriöl., 1911, 58, p. 97 and Münch. Med. Wchnschr., 1909, 49, p. 885.

¹⁸ Ztschr. f. Hyg. u. Infektionskrankh., 1913, 74, p. 61.

REACTIONS OF TRANSPLANTS FROM TWELVE ISOLATED COLONIES ON PLAIN AGAR INTO MILK TUBES OF SAME LOT

	Milk Reaction		
	On 1st Day	On 16th Day	On 30th Day
1. Smooth colony.....	P _H 6.8	P _H 6.2	6.2
2. Smooth colony.....	P _H 6.8	P _H 6.2	6.2
3. Smooth colony.....	P _H 6.8	P _H 7.7	7.8
4. Smooth colony.....	P _H 6.8	P _H 7.7	7.7
5. Smooth colony.....	P _H 6.8	P _H 7.7	8.0
6. Smooth colony.....	P _H 6.8	P _H 6.2	6.2
7. Smooth colony.....	P _H 6.8	P _H 7.7	8.5
8. Smooth colony.....	P _H 6.8	P _H 7.3	7.3
9. Vine leaf like colony.....	P _H 6.8	P _H 6.8	6.8
10. Vine leaf like colony.....	P _H 6.8	P _H 6.8	7.0
11. Lobulated colony.....	P _H 6.8	P _H 7.5	7.7
12. Lobulated colony.....	P _H 6.8	P _H 6.2	6.2
Strain "I 75." One vine leaf colony.....	P _H 6.8	P _H 8.5	9.0
			gelatinized

The differences in the final P_H⁺ reaction are merely the result of differences in the rate of multiplication of the selected colonies. Strain "I 75" in comparison with strain 49 possesses a more rapid growth rate, which in turn gives rise to alkaline split products in a correspondingly shorter time interval. It is as yet undecided, whether the alkaline reactions were caused primarily by the oxidation of the salts of citric acid to alkaline carbonates as recently suggested by Ayers, Rupp and Johnson¹⁹ or the result of a true alkali fermentation. Some incomplete data at our disposal support strongly the contention of the workers mentioned.

Bradley,²⁰ Krumwiede, Pratt and Kohn²¹ and recently Jordan²² pointed out that the differences in the milk reaction of the various paratyphoid strains are probably due merely to a difference in the rate of multiplication. A similar mechanism seems operative in the milk reactions of the typical and the so-called "blue typhoids." Even different members of one and the same strain may develop in milk rapid or slow alkali-producing offsprings. Bromcresol purple milk can therefore not be recommended as a suitable differential medium for the classification of irregular strains.

Indol Production, Methyl Red Test and Hemolysis.—In one tube of Witte's peptone solution inoculated with the second generation of strain "I 75" a slight but definite indol reaction was noted with Ehrlich's reagent on the sixth day. Subsequent tests, including strain "Chr. 76," gave negative results. Through the observations of Andrejew²³ and Bull and Pritchett²⁴ it is known that irregular typhoid strains occasionally produce traces or even large amounts of indol. The methyl red test was positive. All 3 strains failed to produce hemolysis on blood-agar plates.

Reduction of Dyes.—Neutral red in 0.5% agar with or without glucose was not reduced by the 3 strains under discussion. A slight slow reduction (5 days) of malachite green and orcein agar was observed, but also constantly noted with the control typhoid strains.

¹⁹ U. S. Depart. Agr. Bull., 782, 1919.

²⁰ Jour. and Proc. Roy. Soc. N. S. Wales, 1912, 46, p. 74.

²¹ Jour. Med. Research, 1916, 35, p. 55.

²² Jour. Infect. Dis., 1918, 22, p. 511.

²³ Arb. K. Gsndttsamte, 1910, 33, p. 363.

²⁴ Jour. Exper. Med., 1916, 24, p. 39.

The detailed findings may be briefly summarized: Biochemically, strain "I 75" and "Chr 76" differ from the true typhoid strains, including strain No. 49, by their intensified carbohydrate reactions; dulcitol, rhamnose and arabinose broth in the order stated are acidified in a shorter time interval than is customarily recorded for *B. typhosus*. This accelerated ferment action may also be in part responsible for the rapid alkali production in milk, which we found apparently was the result of a more rapid and more intense growth in this medium than is ordinarily observed with typical typhoid strains. Both strains fail to produce papillae on raffinose agar, but do so on rhamnose plates.

SEROLOGIC IDENTIFICATION

A suspension of the gram-negative, motile bacilli, which grew in the blood culture of case 1, was promptly clumped by a polyvalent typhoid immune serum in a slide and in a macroscopic agglutination test. Additional determinations made with specific rabbit antisera and suspensions of living bacteria of strain "I 75" placed this organism serologically with the typhoid bacillus. The reactions were always well marked with typhoid serum and coreactions with other serums were slight or absent. In the course of several months, when an attempt was made to determine more closely the group relationship of strain "I 75" it was found that this organism when preserved in a 0.1% formalinized salt solution was inagglutinable by *B. typhosus* serum, but had apparently acquired the property of being specifically clumped by *B. enteritidis* serums in maximum dilutions. This striking specificity of the killed in contrast to the living suspensions has been occasionally noted with other strains, but never to such a degree as was constantly encountered in the tests with strain "I 75." In the literature we found only the statements by Kafka,²⁵ Klemens,²⁶ Minelli²⁷ and others, that formalinized suspensions may show complete absence or marked reduction of coagglutination reactions. On the other hand, most writers agree that agglutination reactions with living bacteria must be considered more sensitive than those conducted with dead cultures. It is, however, evident that little attention has been paid to this phenomenon and a careful study of immunologic and physicochemical factors responsible for this differences suggest themselves. Repeated tests conducted during the last 8 months with at least 20 different formalinized and living suspensions always gave identical and uniform results, which are shown in table 3.

Strain "Chr. 76" was hyperagglutinable when first isolated and could only be tested after 30 successive transplantations on neutral peptic digest agar. By changing the electrolyte contents of the suspensions and serum dilutions, in using a 0.25% salt solution according to the method of Verzar²⁸ specific reactions and slight coreactions occurred with typhoid serum and living suspensions. These preliminary tests placed, in our opinion, strain "Chr. 76" with the typhoid bacillus. Several months after the date of isolation when absorption tests were in the process of preparation agglutination tests with formalin-

²⁵ Centralbl. f. Bakteriöl., 1906, 40, p. 247.

²⁶ Berl. klin. Wchnschr., 1905, 42, p. 1269.

²⁷ Centralbl. f. Bakteriöl., 1906, 41, p. 583.

²⁸ Centralbl. f. Bakteriöl., 1917, 80, p. 161.

ized suspensions were undertaken. Again the same phenomenon, as already described for strain "I 75," became apparent, namely strain "Chr. 76" was inagglutinable in formalinized killed suspensions; it was, however, specifically

TABLE 3
AGGLUTINATION REACTIONS

Antiserums	Antigen "I 75"		Antigen "Chr. 76"		Antigen "49"	
	Living Second Generation	Formalinized 0.1% 21st Generation	(Living Second Hyperagglutinable) 15th Generation in 0.25% Saline Specific Agglutination	Formalinized 0.1% 30th Generation	Living Second Generation	Formalinized 0.1%
Polyvalent B. typhosus (1:40,000)	1:600 (blood broth) (bile broth)	0	1:1000+++	0	1:20,000	1:40,000
Hooker's Group I	B. typhosus "9" (1:10,000)	1:2000 (20th gener.)	0	1:1000+++	0	1:10,000 (10th gener.)
	B. typhosus "11" (1:10,000)	1:200+++ (20th gener.)	0	1:10,000 (10th gener.)
Hooker's Group II	B. typhosus "Rawlings" (1:8,000)	1:600+++ (20th gener.)	0	1:600++	0	1:8,000 (10th gener.)
	B. typhosus "Dorset" (1:20,000)	1:400+++ (20th gener.)	0	1:20,000 (10th gener.)
Hooker's Group III	B. typhosus "Hopkins" (1:10,000+++)	1:600+++ (20th gener.)	0	1:600+++	0	1:10,000 (10th gener.)
	B. typhosus "1" (1:6,000+++)	1:200 (20th gener.)	0	1:400+++	0	1:6,000 (10th gener.)
B. paratyphosus B polyvalent (1:20,000)	1:20+++	0	1:80++	0	<1:200	0
B. paratyphosus B human (1:10,000)	0	<1:100	0
B. paratyphosus B avian (1:20,000)	1:50±	0
B. paratyphosus B rodent (1:20,000)	0	0
B. paratyphosus A (1:40,000)	1:40+++	0	1:800+++	0	<1:100	0
B. sanguinarum (1:1,000)	1:60+	1:200	0	1:100++	0	1:200
B. pullorum (1:2,000)	1:100	0
B. enteritidis human III (1:20,000)	1:2,000	1:2,000	<1:100	0
B. enteritidis rodent I (1:10,000)	<1:50	1:2,000	1:2,000	"
B. enteritidis calf. (1:10,000)	1:2,000	1:2,000	0
Normal rabbit serum	1:10	0	1:120	0	<1:10	0

agglutinated by B. enteritidis-serums. Strain "Chr. 76" in living suspensions is somewhat more readily sedimented and clumped by typhoid and paratyphoid serums than strain "I 75," but from the standpoint of the serologic data presented in table 4 the two strains must be considered as identical. The parasitic strains differ from the saprophytic ones by their ability of being readily agglutinated by B. enteritidis serums. Strain No. 49 behaves serologically like a

typhoid bacillus. No changes in agglutinability have been noted during the last 4 months. Standardized suspensions are specifically agglutinated by typhoid serums and group reactions are only noted with *B. sanguinarum*-serums.

The fact that formalinized killed suspensions of strain "I 75" and "Chr. 76" were not agglutinated by typhoid serums made the original diagnosis rather questionable and it was thought possible to determine the exact position of the bacteria under consideration by the use of a specific serum prepared with strain "I 75." On account of the high toxicity of this strain we succeeded only after many attempts in producing a highly specific and potent serum of a titer of 1:200,000.

TABLE 4
TESTS WITH STRAIN "I 75" ANTISERUM

Antiserum for Strain "I 75"		Living Suspended 0.1% Formalinized Salt Solution	Killed in 0.1% Formalinized Salt Solution
Strain "I 75".....		1:200,000	1:20,000
Strain "Chr. 76".....		1:100,000	1:80,000
Strain "Sch. 49".....		1:1,000+++;	0
		1:2,000++	
B. typhosus "Rusk".....		1:4,000+++	0
B. typhosus "Blair".....		1:10,000+++	—
B. typhosus "Singleton".....		1:6,000+++	0
B. typhosus "Jacobs".....		1:200+++	—
B. typhosus "Blunt".....		1:1,000+++	—
B. typhosus "Moffitt".....		1:200+++	—
B. typhosus "Kleeberg".....		1:6,000+++	—
B. typhosus "Houston".....		1:1,000+++	—
B. typhosus "Cordona".....		1:8,000+++	—
B. typhosus "15".....		1:4,000+++	—
B. typhosus "Kearney".....		1:4,000+++	0
B. typhosus "52".....		1:200++	0
Hooker's Group I	B. typhosus "40".....	1:2,000+++	0
	B. typhosus "11".....	1:200	0
Hooker's Group II	B. typhosus "Dorset".....	1:2,000+++;	0
		1:4,000++	
Hooker's Group II	B. typhosus "Rawlings".....	1:6,000+++;	0
		1:8,000++	
Hooker's Group III	B. typhosus "1".....	1:2,000+++;	0
		1:10,000++	
Hooker's Group III	B. typhosus "3".....	1:4,000+++;	0
		1:6,000+	
B. paratyphosus "Human 26".....		1:200+++	0
B. paratyphosus A "13, 15, 16".....		1:400+++	0
		1:1,000+	
B. sanguinarum "5".....		1:100+++	0
		1:4,000++	
B. enteritidis, strain 1, origin "rat".....		1:200,000	1:20,000+++
B. enteritidis, strain 2, origin "A. M. N. H., unknown".....		1:200,000	1:20,000+++
B. enteritidis, strain 3, origin "Strassburg, human".....		1:200,000	1:10,000+++
B. enteritidis, strain 6.....		>1:200,000	1:40,000+++
B. enteritidis, strain 13, origin "Califcours".....		1:200,000	1:20,000+++

It is clearly indicated that such a serum gave with living suspensions of a variety of typhoid and also paratyphoid A bacilli, pronounced and fairly uniform coreactions. On the other hand, the "I 75" antiserum agglutinated in formalinized suspensions only its own organism, strain "Chr. 76" and a number of *B. enteritidis* strains isolated from various sources. The coreactions obtained with the 11 typhoid strains, which are under suspicion of having been the sources for the infection of case 1, and the creation of strain "I 75"

were not sufficiently striking to stigmatize any particular one as being antigenically closely related to strain "I 75," and again, the strains which represent the 3 groups of Hooker's classification²⁹ are not influenced serologically by the "I 75" immune serum in such degrees that a relationship of our strain "I 75" to either one of these groups could be arbitrarily deducted. To be sure, the reactions appear more as group reactions which apparently embrace the entire typhoid-paratyphoid group.

At this stage of the serologic identification it was considered necessary to apply absorption tests, naturally using living suspensions as antigens, and absorbing the immune serum completely of their agglutinin content. Extensive experimental series with the organisms of the *B. paratyphosus* and *B. melitensis* groups have convinced us that only the complete removal of all immune substances will give comparable results. With highly potent serums, such as the one prepared with "I 75," the procedure of removal is very tedious; 4 to 8 saturations with living organisms are sometimes necessary to deprive the serum of its entire agglutinin content for the absorbing antigen. The technic used by us is similar to the one described by Taylor, a detailed account therefore appears superfluous.

TABLE 5
ANTISERUMS AGGLUTINATE WITH LIVING ANTIGENS AFTER COMPLETE ABSORPTION

Strains	"I 75" with "I 75"	"I 75" with <i>B. typhosus</i> "9"	"I 75" with "Rawlings"	"I 75" with <i>B. typhosus</i> "3"	<i>B. typhosus</i> "Rawlings" with "I 75"	<i>B. typhosus</i> "3" with "I 75"	<i>B. typhosus</i> "Rawlings" with <i>B. typhosus</i> "3"
I "75".....	0	1:200,000	1:200,000	1:200,000	0	0	0
Chr. "76".....	0	1:100,000	1:100,000	1:100,000	0	1:40±	0
No. 49.....	0	1:40±	0	1:320	1:10,000	>1:5,000	1:40
Hooker's Group I							
<i>B. typhosus</i> "9".....	0	0	0	1:40+	1:4,000	1:2,000	1:80
<i>B. typhosus</i> "11".....	0	0	0	1:80	1:4,000	1:600	1:200
Hooker's Group II							
<i>B. typhosus</i> "Rawlings".....	0	1:40±	0	1:320	1:10,000	1:2,000	1:600
<i>B. typhosus</i> "Dorset".....	0	1:320	0	1:160	1:2,000	1:2,000	1:40
Hooker's Group III							
<i>B. typhosus</i> "Hopkins".....	0	1:160	1:40	1:320	1:4,000	1:4,000	1:80±
<i>B. typhosus</i> "1".....	0	1:320	1:640	1:640	>1:2,000	>1:5,000	0
<i>B. typhosus</i> "3".....	0	1:320	0	1:160	1:2,000	0

0 indicates agglutination less than 1:40.

Strain "I 75" removes from its own immune serum all coagglutinins for the *B. typhosus*. On the other hand, a typical *B. typhosus* recently isolated and belonging to group I of Hooker's classifications removes from the serum of strain "I 75" the immune substances for his own strain and closely allied representatives of group II and III. The agglutinins for strain "I 75" remain quantitatively intact. A similar phenomenon takes place when this serum is absorbed with representatives of groups II and III. It is, however, apparent that a *B. typhosus* strain belonging to group III deprives the immune serum "I 75" in repeated tests incompletely of its agglutinins for the representatives of groups I, II and III. From the studies of Hooker the rather heterogeneous composition of this group is known and irregular reactions actually characterize this subgroup of typhoid bacilli. In a typhoid immune serum prepared with

²⁹ Jour. Immunol., 1917, 2, p. 1.

the type strain "Rawlings" group II the strain "I 75" removes its own group agglutinins, but the major typhoid agglutinin remains practically unaltered. The effect of "I 75" on an immune serum prepared with an organism of group III *B. typhosus* 3 is identical, and again, a *B. typhosus* of group III removes from a group II serum not only the agglutinins for his own group, but also those of strain "I 75" and group I simultaneously, thereby reducing the active substances for group II. These preliminary absorption tests will be enhanced along various other, particularly quantitative, lines as suggested in the recent publication by Andrewes and Inman,³⁰ but they are, so far as it concerns typhoid serums, sufficiently definite to draw certain deductions. Speaking in terms of agglutinin content of these serums, it is evident that a strain "I 75" serum contains, aside from its own major agglutinin, coagglutinins for groups I, II and III of Hooker's classification and absorption with representatives of these groups removes only these group-agglutinins. A "Rawlings" or a group III serum, on the other hand, has group agglutinins for "I 75" which can be specifically absorbed by this strain. Agglutinins for group III in a group II serum remove also the immune substances for strain "I 75."

Serologically strain "I 75" and "Chr. 76" belong to the typhoid group of bacteria; they differ antigenically from the three groups of Hooker; but are closely related to his heterogeneous group III. We are unfortunately not in possession of the typhoid strains used by Weiss³¹ and therefore cannot state in which antigenic subgroup mentioned in his study strain "I 75" and "Chr. 76" should be placed. One point is certain: Our strains stand apart as a definitely differentiable type, even when using living cultures. Moreover, their relation to the typhoid group was shown only by the use of living suspension; formalinized antigens were either inagglutinable or highly specific.

Attention has already been called to the interesting fact that strain "I 75" and "Chr. 76" gradually acquired the ability to be agglutinated in living, and in killed suspensions as well, by *B. enteritidis* serums and vice versa. This group of organisms was uniformly agglutinated to the titer limit by the specific "I 75" immune serums. On the other hand, strain 49 repeatedly tested was not agglutinated by any of the available *B. enteritidis* serum. At first the observation was explained by the well-known fact that *B. enteritidis* serums and vice versa *B. typhosus* serums in many instances give striking coreactions. Already Durham³² and later Kutscher and Meinecke,³³ Liefmann³⁴ and others called attention to this peculiar serologic relationship of certain *B. enteritidis* strains to *B. typhosus*. Absorption tests, however, separated the two organisms in a decisive manner. Thus it would appear to be a simple procedure to determine whether our strains are true *B. typhosus* or true *B. enteritidis*. Our absorptions test produced, however, paradoxical results (see table 3).

Complete removal of the *B. enteritidis* agglutinin in a "I 75" immune serum deprives this serum also of the same substances for strain "I 75" and again a *B. enteritidis* serum absorbed with the irregular strain "I 75" or "Chr. 76" fails to agglutinate all of the *B. enteritidis* strains tested. Judging from these paradoxical results, which were repeated with various other completely and incompletely absorbed serums, we should conclude that strain "I 75" and "Chr. 76" are typical nongas-producing *B. enteritidis* strains. We searched in vain for similar observations in the literature, but could only find the references

³⁰ Medical Research Committee, Special Report, Series, No. 42, 1919.

³¹ Jour. Med. Research, 1917, 31, p. 135.

³² Lancet, 1898, 1, p. 154 and Brit. Med. Jour., 1898, 2, p. 588.

³³ Ztschr. f. Hyg. u. Infektionskrankh., 1906, 52, p. 30.

³⁴ München. med. Wchnschr., 1908, 55, p. 159.

already mentioned. Christiansen,³⁵ the only writer who is thoroughly familiar with the nongas-producing *B. enteritidis* or paracolony strains, failed to conduct absorption tests probably because his serum coagglutinated typhoid bacilli in dilutions, which did not suggest such procedures. Until extensive studies with a large number of *B. typhosus* and *B. enteritidis* strains have demonstrated the antigenic relationship of these bacteria, we only record our observations and abstain for the present from offering an explanation. It is not unlikely that the *B. enteritidis* coreaction is characteristic for atypical typhoid strains and in this respect may have considerable diagnostic value and may even strengthen our conception of the typhoid nature of strain "I 75" and "Chr. 76."

In this connection attention is directed to the observations of Sobernheim and Seligmann,³⁶ which indicate a peculiarly marked lability of the antigenic properties of many *B. enteritidis* strains. Two old laboratory strains of this organism showed a transformation of their biologic properties, which was frequently combined with changes in the cultural characteristic. Careful plating methods demonstrated a number of daughter colonies, which apparently represented the transitional stages between the original and the finally transformed irregular strains. These observations are suggestive when we recall that our atypical strains acquired agglutinability for *B. enteritidis* serums in the course of a saprophytic life on agar slants. Neither the biochemical functions of our strains, nor the susceptibility for specific agglutination with typhoid serums have, however, changed in the course of at least 150 transplants. This and similar observations have convinced us that all future publications on pathogenic micro-organisms should definitely state whether the biologic and biochemical studies recorded were made on parasitic or saprophytic offsprings of the original culture.

Identification by Pathogenicity and Protection Experiments.—The freshly isolated strain "I 75" was exceedingly toxic for rabbits; the symptoms and anatomic findings differed in no respect from those commonly seen in animals intoxicated by true typhoid bacilli.

Guinea-pigs of 250-300 gm. of weight succumbed to intraperitoneal inoculations of from 60-100 million living organisms. Careful immunization with heat-killed organisms even by subcutaneous application of the inoculum is difficult; about 50% of the guinea-pigs show progressive emaciation without organic changes or lesions commonly noted in paratyphoid infections. Rats fed for one entire week with broth culture of "I 75" eliminated the fed bacteria, but remained clinically well.

Protection Experiments.—Recent studies conducted in this laboratory and to be published elsewhere demonstrated that the tissues of typhoid immune and nonimmunized rabbits destroy in a given time interval (24-48 hours) approximately the same number of intravenously inoculated typhoid bacilli. On the other hand, paratyphoid immune rabbits can apparently dispose of an infection produced by an intravenous inoculation of paratyphoid organism more rapidly and more completely than the nonimmune animals. This principle was applied to the identification of strain "I 75."

Exper. 1:—On Dec. 16 rabbit 1, which had been intensively immunized with dead and living *B. typhosus* "Rawlings" (agglutination titer of the serum 1:6000), rabbit 2, immunized in an identical manner with the strain "I 75" (agglutination titer of the serum 1:100,000) and a normal rabbit of the same litter and weight (agglutination titer <1:10) were inoculated intravenously with 1 c.c. each containing 6,400 million living organisms of strain "I 75." On

³⁵ *Centralbl. f. Bakteriol.*, 1914, 74, p. 474.

³⁶ *Deutsch. med. Wchnschr.*, 1910, 36, p. 351.

Dec. 17, seventy-four hours after the injection of the infective dose, rabbit 3 was profoundly intoxicated and showed rapid breathing and diarrhea. The two immune animals appeared less active and ate little. All three rabbits were exsanguinated under ether; the organs were removed aseptically and portions of the same were pulped with sand and saline in sterile mortars and diluted in such proportions, that each cubic centimeter of saline contained 100 mg. of tissue pulp. This material was plated as dilutions in peptic digest agar. The plates were counted after 48 hours' incubation at 37 C. Table 6 illustrates the average number of viable bacteria demonstrated in the tissues and in the blood stream.

TABLE 6

EXPERIMENT I: INTRAVENOUS INJECTION OF 6,400 MILLION ORGANISMS. SACRIFICED AND TISSUES PLATED 24 HOURS AFTER INJECTION

Tissues	Rabbit 1 Immune to B. ty- phosus "Rawlings"	Rabbit 2 Immune to Strain I 75	Rabbit 3 Normal
Agglutination titer.....	1:6,000+++	1:100,000+++	<1:10
	Per 100 mgm. of tis- sue. The following Grew After 24 Hours' Incubation	Per 100 mgm. of tis- sue. The following Grew After 24 Hours' Incubation	Per 100 mgm. of tis- sue. The following Grew After 24 Hours' Incubation
Liver, left and center lobe.....	12,000	20,000	480,000
Liver, right and center lobe.....	30,000	15,000	720,000
Bile.....	3 per 0.8 c c	0	238,000,000 per 1.7 c c
Gallbladder wall.....	90	0	18,000
Spleen.....	380,000	110,000	6,300,000
Bonemarrow.....	13,200	70,000	1,680,000
Mesenteric lymphnodes.....	180	400	12,400
Kidneys.....	72	6,700	34,000
Lungs.....	17,500	1,000	640,000
Heart blood.....	2,400	52,000
Carotis blood.....	200	48,000
Duodenum.....	Negative for B. I 75	1 colony of B. I 75	20 colonies of I 75
Ileum.....	150 colonies per loopful of intes- tinal content	2 colonies of B. I 75	

It is quite evident that the normal animal is less readily capable of destroying the intravenously inoculated bacteria of strain "I 75" than the immune one. The profound intoxication is indicated by a high bacterial count of the bone marrow, an observation which has recently been emphasized by J. T. Parker. The bile and spleen are also heavily infected. On the other hand, there is little difference between the animal immunized against the infecting strain and the one protected against the "Rawlings" organism. Both rabbits are destroying the inoculated organisms in the chosen time interval of 24 hours in approximately equal proportions. From our extensive experience with this particular method of immunity research already referred to we are justified in concluding that strain "I 75" behaves in the immune and normal rabbit like a paratyphoid organism, but that apparently no differences exist between the destructive forces of the animal immune to the infective strain "I 75" and the one which is only protected against the type typhoid strain "Rawlings." This experiment again supports the contention that strain "I 75" is antigenically closely related to the typhoid bacillus.

Bull and Pritchett²⁴ and recently J. T. Parker²⁷ have emphasized the fact that rabbits immunized with typhoid bacilli are highly and specifically resistant to intoxication with this organism. They withstand, as a rule, from 30 to 40 lethal doses of the living bacilli. Unfortunately no experimental data are available which prove conclusively that the toxic substances derived from organisms of the typhoid-paratyphoid group are strictly specific and our tests along these lines have not sufficiently matured to enable us to express a final

²⁷ Jour. Med. Research, 1919, 39, p. 301.

opinion. It is therefore with some hesitancy that we record some protection tests, which in themselves are very suggestive and which should encourage further inquiry along these lines.

Exper. 2.—One rabbit immune strain "I 75." 2 rabbits to different strains of typical *B. typhosus*, 1 rabbit to *B. paratyphosus* B, 1 to *B. coli* and 2 controls were injected with 50 lethal doses of strains "I 75." The animals succumbed after the following time intervals:

TIME IN WHICH ANIMALS SUCCEMBED TO LETHAL DOSES

	Died
Normal rabbit.....	3 hours, 50 minutes after the injection
Normal rabbit.....	4 hours, 10 minutes after the injection
Immune to <i>B. paratyphosus</i>	5 hours, 5 minutes after the injection
Immune to <i>B. coli</i>	5 hours, 25 minutes after the injection
Immune to <i>B. typhosus</i> polyvalent.....	23 hours after the injection
Immune to <i>B. typhosus</i> polyvalent.....	26 hours after the injection
Immune to Strain "I 75".....	36 hours after the injection

It is a known fact, that normal rabbits vary considerably in their resistance to bacterial toxins of the typhoid group. This in part explains the unfortunate use of an intoxicating dose which also proved fatal in the specifically immune rabbit. The results could therefore be made more definite, but at least they indicate, as presented, that true typhoid bacilli protect to a certain degree against strain "I 75" and that paratyphoid and colon immune rabbits succumb to the intoxication as readily as the nonimmune ones.

We also attempted in a series of immune and normal guinea-pigs to determine the distribution and destruction of strain "I 75." Thus far we have noted exceedingly interesting paradoxical results, namely, the specifically and the typhoid immune guinea-pigs succumbed to the infection in contradistinction to the normal animals which remained alive. Until we have sufficiently often repeated these observations and can offer an explanation for this phenomenon we are withholding for the present the citation of a detailed experiment.

DISCUSSION

Before entering into a discussion of the various problems that suggest themselves in the analysis of the data, it is necessary to present a summary of the events as they appear in our interpretation.

An animal caretaker, who had intimate contact with secreta and cadavers of typhoid experimental animals, contracted an abortive attack of typhoidal fever. The short duration of his illness is probably due to his age immunity and to the prophylactic vaccination which had been administered to him, one and one half years previous to his infection. On only one occasion was an irregular typhoid strain isolated from his blood stream. His Widal reaction and stool and urine cultures were always negative. About two months later a vaccinated laboratory worker, who was experimenting with the irregular strain and also with the cultures of *B. typhosus* used for our experimental work on animals, developed a severe attack of typhoid fever. An irregular strain of *B. typhosus* identical with the strain isolated from

the caretaker was demonstrated on one occasion in her urine. Repeated blood, stool and urine culture during the relapse periods were negative. Her unvaccinated aged father contracted typhoid fever twelve days after handling a wooden spatulum, which had been used by the patient to prepare her stool specimen for shipment to laboratory. The stool and urine of this case contained typical typhoid bacilli. From the standpoint of the bacteriologist the variants of the *B. typhosus* may have originated in the following manner: The highly immune caretaker atavistically changed one or several typical strains of *B. typhosus* to the irregular strain described as "I 75," which in time caused a second severe infection in a vaccinated young woman. Her strain in passing through the tissues of an old, nonimmunized man reverted to a typical *B. typhosus*.

The observations recorded in the foregoing paragraphs deserve, however, a more detailed consideration from three different points of view, namely: (1) epidemiologic, (2) clinical and (3) bacteriologic.

1. *Epidemiology*.—As already outlined in the history of case 1, we feel convinced on epidemiologic grounds that our animal caretaker contracted his infection through intimate contact with heavily infected typhoid secreta of rabbits and guinea-pigs. Most painstaking inquiries which were met by a liberal cooperation on the part of our janitor failed to reveal any possibility of outside connections with acute, latent or carrier typhoid fever cases. His whereabouts the last four months previous to his transitory illness were readily traceable on account of the illness of his wife and his compulsory functions as a nurse. The only determinable source was the infection from our experimental animals. It is well known to workers in this field of experimental pathology that renal (in the first) and gallbladder carrier rabbits (in the second place), as a rule, may shed enormous numbers of living, virulent typhoid bacilli. The sawdust bed of such carrier cages regularly contains demonstrable *B. typhosus*, and again the handling of typhoid animal cadavers is connected with even greater danger of exposure. Even with the average amount of care it is unavoidable that such material soil the hands of the cleaner or cremator. For this reason it has been our policy to protect our personnel by vaccination, repeated in from one to two years. Kisskalt,³⁸ in his recent summary of typhoid laboratory infections, mentions several cases which resulted from contact with animal material, particularly rabbit typhoid carriers.

³⁸ Ztschr. f. Hyg. u. Infektionskrankh., 1915, 80, p. 145.

One case is cited in which a laboratory janitor contracted typhoid fever through eating his meals in a stable where a goat had been inoculated with living typhoid bacilli. Thus it is apparently not an uncommon occurrence that such experimental material serves as a potent source for enteric fever infections. It may, however, be stated that undoubtedly some as yet unknown factors must be concerned, because the writers have in the last four years repeatedly aspirated or otherwise come in contact with heavily infected animal material, and neither of the two has contracted typhoid fever. It is our belief that the prophylactic vaccination practiced every 12 months is mainly responsible for the fortunate outcome of the unavoidable accidents.

Epidemiologically, case 2 is again explained on circumstantial evidence only. The patient is personally convinced that she contracted the infection in the course of her laboratory work in making innumerable slide agglutination tests. It is difficult, however, to state the date on which she probably became infected and particularly in the light of the interesting bacteriologic findings, it would be invaluable to know whether she infected herself with strain "I 75" isolated from case 1, or with one of the many typhoid strains in use at the time theoretically accountable for her illness. One thing is certain, that the identical organisms were tested by her which are considered responsible for case 1. An infection outside of the laboratory has been completely ruled out by a searching study of a possible source. Her first clinical symptoms developed about 50 days after she had handled strain "I 75," according to her records. There are two possibilities which may help to explain the identity of the strain "Chr. 76" isolated from case 2 with strain "I 75," either in the vaccinated person strain "I 75" remained latent or one or several of the experimental strains of *B. typhosus* were in her tissue transformed in a similar manner into an atypical strain as we assume it to be case for strain "I 75." Personally, we believe that the worker handled the culture "I 75" perhaps unknown to herself in a period shortly before she developed the clinical signs of typhoid fever. Repeated attempts to prove experimentally or otherwise the suggested explanation have failed and the identity of culture "I 75" and "Chr. 76" is the only tangible link which establishes the connection of case 2 with case 1.

The practical epidemiologist has no difficulty in explaining case 3. The handling of a spatulum used for the preparation in a stool specimen derived from a clinical typical typhoid fever case furnishes the connecting bridge between cases 2 and 3, and again, the daily presence

of a physician in the house of patient 2 establishes beyond any doubt the incubation time of 12 days for case 3; this fact would therefore be additional proof of the correctness of the deductions above stated. Such conclusions, however, appear doubtful to the bacteriologist when he notices striking cultural and serologic differences in the offending organisms of cases 2 and 3, and it is only natural to suspect that case 3 was the result of an outside instead of an inside house contact infection. One of us (N. M. N.) reviewed epidemiologically in detail the various typhoid cases which were known to exist in the community, and which were thought to be reasonable sources of infection. It was not only possible to prove conclusively that the infection did not originate from any one of the outside cases, but the members of the household also unanimously agreed that Sch. had not taken any meals outside of his house during the illness of his daughter. While the bacteriologic findings suggest a new source of infection, all the epidemiologic data prove conclusively a contact infection. The importance of this fact will become apparent in connection with the discussion of the bacteriologic findings.

2. *Clinical Data.*—The clinical findings in case 1 were indefinite, and without a positive blood culture an early accurate diagnosis would have been impossible. Also in case 2 the course of the first febrile attack was mild and clinical diagnosis was only ventured during the relapse. It is not unlikely that this infection would have ended abortively had the patient remained in bed a few days longer. In neither case were rose spots observed, nor was the spleen definitely palpable. The Widal reactions were negative, and even in applying Dreyer's principle, exceedingly doubtful. Even the blood culture method, which, according to recent accounts of Eggerth,³⁹ is the most reliable procedure for the diagnosis of typhoid fever infections in the vaccinated, failed completely. A typical leukopenia and a slow pulse were, however, suggestive of such a disease. Stool and urine examinations of case 1 were negative. In case 2 one urine specimen contained irregular typhoid bacilli. Negative stool specimens were to be expected on account of the carbohydrate diet, which as Torrey⁴⁰ and we have repeatedly shown, reduces the viable *B. typhosus* to such a degree that a bacteriologic demonstration is frequently impossible even with brilliant green eosin plates.

³⁹ Jour. Infect. Dis., 1919, 25, p. 166.

⁴⁰ Ibid., 1915, 16, p. 72.

These observations confirm the facts that have been established by the medical service of the U. S. Army and recently presented by Soper ⁴¹ at the meeting of the American Public Health Association, namely, typhoid fever in the vaccinated may as a rule run a mild course and one difficult to recognize. Clinically, it is also impossible to differentiate a paratyphoid from a typhoid infection. It therefore could be suggested by our critics that in the light of the serologic tests the infections were caused by a nongas-producing *B. enteritidis* or *B. paratyphosus*. The clinical observations lend little support to this contention. The *B. enteritidis* infections thus far reported by Jochmann ⁴² and observed by one of us, are always abrupt and in their initial symptoms governed by marked gastro-intestinal reactions. Furthermore, relapses as Torrens and Whittington ⁴³ and Jochmann have pointed out, are comparatively rare and usually of shorter duration than in true typhoid fever.

It may be a mere coincidence when Kisskalt ³⁷ states in his summary that in laboratory infections the Widal reaction is frequently negative. Concerning this point accurate data determined by the macroscopic agglutination test should be collected from future cases.

The diagnosis of typhoid fever in the vaccinated was apparently connected with difficulties in the Army, otherwise a classification into (1) suspected (2) clinically and (3) bacteriologically proved cases would not have been advocated. Little comment is necessary when dealing with the clinical aspect of case 3; the symptoms and bacteriologic findings were typical in every respect.

3. *Bacteriologic Findings*.—Our bacteriologic findings with one cell cultures have established the following facts: Strain "I 75" and "Chr. 76" isolated from cases 1 and 2, respectively, behave as irregular, atypical typhoid bacilli. Strain 49 isolated from case 3 is a typical *B. typhosus*. The irregular strains exhibited the following variants: They are rapid dulcitate, rhamnase and irregular arabinose fermenters; they are "blue typhoids" producing rapidly alkalies in bromocresol purple milk; one strain produced indol in the second generation; serologically, they are hyperagglutinable as living organisms and belong to a subgroup of group III of Hooker's typhoid classification. They are typhoid-inagglutinable in formalinized suspensions, but are as saprophytic strains agglutinated by *B. enteritidis* serums and by their

⁴¹ Am. Jour. Pub. Health, 1920, 10, p. 301.

⁴² Lehrbuch d. Infektionskrankheiten, Berlin, 1914, p. 85.

⁴³ Brit. Med. Jour., 1915, 2, p. 697.

own with immune serums. Typhoid immune rabbits are protected against the infection and intoxication by the irregular strain. These variants, with the exception of an acquired agglutinability for *B. enteritidis* serums, have remained constant in the course of at least 150 transplants on peptic digest agar. It is evident, that the irregular strains differed bacteriologically and serologically only in degree from the true *B. typhosus*. Inherent properties, like certain carbohydrate fermentations (rapid fermenters) and coreactions with *B. enteritidis* serums, are enhanced to a marked degree of activity, but there are no suggestions of true mutation, a conception which is well supported by the masterly analysis of the available facts in Eisenberg's⁴⁴ summary on "Bacterial mutation." This paper contains all the important references to atypical typhoid or paratyphoid strains, but we have been unable to identify our organisms with any one of the hitherto described irregular bacteria of the typhoid paratyphoid group. In many instances the method of identification has been so incomplete that it would be mere guess-work even to correlate the organisms recently described by Guerbet and Henry, Faroy, Lafforgue, Messerschmidt, Marotte, Fromme, Oette, Wagner, Goebel, Ohno, Wille, Broughton-Alcock³ and others with our own.

Irregular fermentation reactions are frequently accompanied by similar variations in the serologic behavior. Inagglutinable and peculiarly receptive strains have been repeatedly described, but an analogue to our observation was not found in the references at our disposal. It is, however, emphasized that agglutinability is a variable characteristic (Henderson Smith⁴⁵), and that it should be used with caution in the differentiation of closely allied variants of the typhoid-colon groups.

It would be interesting, indeed, to know how this intensification of the inherent properties was induced and how far the resultant variants influence the pathogenicity and epidemiology of the disease, and to what degree the occurrence of such variants influences our conception of the homogeneity of the *B. typhosus* group. Is it a mere coincidence that the progressively atypical and irregular strains occurred in two typhoid vaccinated persons and apparently the same strain reverted atavistically to a typical typhoid strain in an aged nonvaccinated man? We are not in a position to answer these questions, because test-tube experiments and innumerable rabbit and guinea-pig experiments have

⁴⁴ *Ergebn. Immunitätsforschung*, 1914, 1, p. 28.

⁴⁵ *Trans. Fifteenth Int. Congress of Hyg. and Demogr.*, 1912, 2, p. 99.

not enabled us to accomplish this transformation. Laboratory animals are nonsusceptible to the *B. typhosus*, and even extended latency of this organism in the bile or tissues of rabbits, guinea-pigs, dogs or monkeys has in our experience created only inagglutinable but otherwise typical offsprings. A certain degree of adaptability to changes in the H-ion concentration of the substratum may be noted as was recently pointed out by one of us. But this fact has remained thus far the only tangible suggestion that existence in animal tissues may be conducive to the production of variants. The literature, however, contains sufficient observations which indicate that fermentative properties may be acquired on artificial mediums. *B. typhosus* can, as Twort,⁴⁶ Penfold,¹⁶ R. Müller¹⁵ and others have shown, gradually "mutate" into distinct variants, which, however are inconstant. After a series of cultivations on a substratum free from the enhancing carbohydrate fermentation stimulating substance, the variants as a rule revert to the original ancestral type.

It is quite plausible to assume that such a transformation of one or several true typhoid strains occurred in our case 1. In this connection we cannot overlook the possibility that perhaps animal paratyphoid strains to which the man was exposed underwent transformation. A change of their labile properties is in the realm of possibilities. Such a conception as well as the criticism that we were possibly working with mixed cultures can be readily dismissed. Old and recent observations have proved the low pathogenicity of animal strains of *B. paratyphosus* B and *B. enteritidis*, and again our discussion of the bacteriologic and serologic findings given above lend little support to this assumption. Repeated plating and finally the preparation of one cell cultures by the Burri method were chosen to rule out conclusively the danger of a mixed strain.

Passing through a second vaccinated host the irregular strain undoubtedly met an environment similar to the first, and it therefore preserved the new properties acquired. Only when flourishing in the tissues of an unvaccinated individual the variant reverted for reasons as yet unknown to the original typical *B. typhosus*. In our opinion, the most important question: "Is a change in metabolic activities also accompanied by alterations in pathogenic and antigenic properties?" cannot be answered conclusively. Animal infection and intoxication experiments fail to inform us concerning the true pathogenicity for

⁴⁶ Proc. Roy Soc., London, 1907, B. 79, p. 329.

man, and we are mainly dependent for an explanation on the results of our serologic tests. It was noted that strain "I 75" and "Chr. 76" differed in many respects from the type strain "Rawlings," and that these strains belong to a subgroup of the *B. typhosus*. It is the recognition of this fact that prompted this report and that furnishes some questions of considerable practical importance. The answers to some of these queries can perhaps be best introduced by the citation of a few lines from the recent book of Adami:⁴⁷

Still I shall feel that these pages have not been written in vain if I succeed in drawing increased attention to the fact that the bacteria are organisms acutely susceptible to changes in environment, that as species they are far from presenting constant characteristics, and that to a variability which may impress itself upon a greater or less number of generations is to be ascribed, in part, the differences between successive epidemics, between the successive stage of one epidemic, and between individual cases of disease.

We are well informed concerning the variability of the coccus group; but few authentic observations have thus far been made with micro-organisms of the typhoid group. This is in part the result of incomplete epidemiologic investigations and of abbreviated methods for the identification of the typhoid bacillus which will rarely disclose functional variants and therefore will never lead to the discovery of new types responsible for certain epidemics. As a matter of fact, the epidemiologist of today is frequently considered nothing more than an expert in detective and police methods, when his highest function primarily should consist of the intricate analysis of all the biologic and dynamic forces leading to an epidemic. For this reason he should have at his command a laboratory of his own, and a force of experts fully equipped to study biologically the suspected causes. With the progress of our methods of sanitation and preventive immunization the necessity for the detection of new disease producing variants should be proclaimed with unremitting insistence. Perhaps for no other disease is this demand more urgent than for typhoid fever. Only when these studies have been made on every occasion will it be possible to answer the mooted question: Does the prophylactic vaccination with a monovalent antigen really confer the maximum obtainable protection? It is not our intention to enter into a consideration of this controversy, but the observations of Mock,² Kisskalt³⁷ and our own clearly demonstrate that it is the vaccinated or the laboratory worker, who develops irregular typhoid strains. We should, therefore, insist on a most careful study of the bacterial strains from these sources.

⁴⁷ Medical Contributions to the Study of Evolution, New York, 1918, p. 131.

SUMMARY

This report describes the laboratory infections in a vaccinated caretaker (case 1) exposed to laboratory animals shedding living typhoid bacilli and a laboratory worker also immunized (case 2), who was regularly working with strains of the *B. typhosus* group. The last case apparently caused a severe and fatal house contact infection (case 3). From the blood of case 1 and the urine of case 2, on one occasion only, an irregular, atypical organism, and from the stool and urine of case 3 typical typhoid bacilli were isolated. The irregular typhoid strains ferment without gas production the usual carbohydrates, also dulcitol, rhamnose and irregularly arabinose; they rapidly cause an alkaline reaction in milk, and develop small rhamnose papillae comparatively slowly. In formalinized killed suspensions they are only agglutinated by their own immune serums and recently also by *B. enteritidis* serum. As living organisms they are specifically clumped and sedimented by typhoid immune serums and can be classified by absorption tests with one of the subgroup of group III in Hooker's classification. Antiserums prepared with these organisms agglutinate typhoid bacilli in living suspension only and coagglutinate slightly, if at all, the representatives of the paratyphoid B or A group. As saprophytic strains they behave like nongas-producing strains of *B. enteritidis* from which group they cannot be separated even by careful absorption tests.

The possible bearing of these observations on the epidemiology, clinical aspect and bacteriology of typhoid fever is discussed, and it is suggested that special attention be paid to the occurrence and the detailed study of irregular typhoid strains in the typhoid-vaccinated.

A SPONTANEOUS EPIDEMIC AMONG LABORATORY
RABBITS CAUSED BY A PARATYPHOID
B. BACILLUS RELATED TO
THE RODENT GROUP

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Spontaneous bacterial infections among laboratory or breeding rabbits are not uncommon. Irrespective of the confusing nomenclature,¹ which may at first suggest a variety of microbial causes, the etiology of such epidemics is in our experience usually confined to *B. cuniculisepticus* and *B. bronchisepticus*. Recently our attention was called to the occurrence of true paratyphoid *B.* infections.

According to the available information, epidemics among rabbits caused by this group of organisms are rare. Uhlenhuth and Hübner² mention, in their summary on the paratyphoid group, that Holst and Hottinger determined bacteria of this group to be the cause of spontaneous epizootics among rabbits. Also Pfeiler³ expresses the same idea in a short sentence in one of his recent reviews. A perusal of the articles of Holst and Hottinger referred to did not inform us of the facts on which these statements are based. In his complete review Loele⁴ does not mention Holst and Hottinger. A definite description of paratyphoid fever in rabbits as a disease entity is therefore not available although Morgan⁵ obtained three cultures of an organism like paratyphoid A from rabbits' feces, an observation that we were able to confirm. In 1914 Ferry⁶ made an intensive study of the causative organisms found in *B. bronchisepticus* epidemics among laboratory animals. In a few cases he was able to isolate an organism of the paratyphoid *B. enteritidis* group; he considered these bacteria to be secondary invaders superimposed on an infection with *B. bronchisepticus* or *B. cuniculisepticus*. Krumwiede, Valentine and Kohn⁷ have recently published an article in which they show that paratyphoid strains isolated from guinea-pigs, mice, rabbits and cats differ antigenically based on specific absorption tests from human paratyphoid *B.* or *B. schottmülleri*-strains. A distinct type or group, the so-called "rodent paratyphoid," is according to their views encountered in spontaneous infections of laboratory animals, especially rodents.

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¹ Hutyrá and Marek: *Spezielle Pathologie & Therapie d. Haustiere*, 1913, 1, p. 104; Ferry and Hoskins: *Jour. Lab. & Clin. Med.*, 1920, 5, p. 311.

² Kolle and Wassermann: *Handb. d. pathog. Mikroorganis.*, 1913, 3, p. 1102.

³ Friedberger and Pfeiffer: *Lehrbuch d. Mikrobiologie*, 1919, 2, p. 917.

⁴ *Ergebn. d. allg. Pathol. u. Pathol. Anatomie*, 1915, 18, p. 628.

⁵ *Brit. Med. Jour.*, 1903, 1, p. 1257.

⁶ *Jour. Path. & Bacteriol.*, 1914, 18, p. 445.

⁷ *Jour. Med. Res.*, 1919, 34, p. 449.

In this connection the report of MacConkey* on some cases of food poisoning should also be recalled. This worker isolated a paratyphoid *B. bacillus* from the hind limb of a rabbit, the only part of a meal which had apparently caused three cases of food poisoning and the death of a 6 months old baby. This organism was identical with the nonlactose fermenting bacteria obtained from the intestines of the baby. Certain facts presented by MacConkey make it certain that when purchased the rabbit was fit for human consumption and contamination must have taken place at the consumers' house. The possible importance of the observation of MacConkey will be considered separately more in detail.

In the light of this information it appeared profitable to investigate more carefully the spontaneous epidemic that occurred in our animal house. This study was particularly valuable in furnishing a rabbit-pathogenic paratyphoid bacillus and a number of data, which assisted us materially in the analysis of the factors of immunity operative in experimental typhoid carriers of this species. We shall have occasion to refer to the organisms isolated from our epidemic in other papers and consider for the present only the symptoms, the necropsy and microscopic findings, the organism isolated, its serologic classification in the paratyphoid group and its pathogenicity for various animals.

HISTORY OF OUR EPIDEMIC

Nov. 26, 1918, two lots of rabbits were received in the laboratory. One set came from a reliable dealer; his rabbits had not, during the past two years, shown signs of coccidiosis or "snuffles." The second lot was sent out by a breeder unknown to us, who had been recommended as always having been successful in raising rabbits. When these rabbits were unpacked, one female was found to have diarrhea and signs of a previous abortion. This animal was immediately isolated but had already been in intimate contact with 12 others of the same lot. This visibly diseased rabbit died Nov. 8 and was only superficially examined. No attention was paid to lung lesions and no cultures were taken. Death was attributed to a polybacterial puerperal septicemia, not uncommonly observed in rabbits closely packed and shipped in unsuitable boxes.

Dec. 8 and 9 two rabbits, that had been in contact with this animal, succumbed. Both animals had diarrhea and extensive bilateral croupous pneumonia. Cultures of paratyphoid *B. bacilli* were obtained from the lung tissues of both rabbits. The anatomic lesions did not suggest additional cultures. However, the lung lesions differed in many respects from those ordinarily encountered in our laboratory and indicated, even before we had obtained and studied the cultures, that we were not dealing with one of the common, intercurrent infections. A careful quarantine and a daily inspection of the stock animals was immediately instituted.

A fourth rabbit, rabbit 1371, was found to be sick on Dec. 11. It refused all food, had a purulent nasal discharge and diarrhea, and its coat was shaggy and rough. This large animal, weighing 3150 gm., was chloroformed. The anatomic lesions were typical of those commonly found in experimentally produced paratyphoid infections. Cultures were obtained on direct plating from all the organs, except the bile. The heart blood contained 720 colonies per c.c.

Dec. 13 a fifth rabbit, 1372, was found dead. This animal had not been in contact with the previously mentioned rabbits and had been placed in a single

* Jour. Hyg., 1906, 6, p. 570.

isolation cage of the infectious disease room the day before. It had anatomic lesions similar to 1371. The intestinal sloughs were particularly well marked in the appendix region. Cultures were also obtained from all of the organs. Epidemiologically this animal was probably infected by the careless use of cleaning utensils employed in the removal of the manure. Previous to Dec. 8, when we were not as yet fully informed as to the nature of the disease, the caretaker used the same scraper on the cage holding rabbit 1372 that he had used in cleaning the section in which rabbits 2 and 3 had succumbed. It was quite obvious that only rigorous measures would interrupt the chain of continuous contact infections. Thorough disinfection of all the suspected cages with lysol and lime, sterilization of the cleaning utensils, food and drinking cups accomplished the desired result. Further cases have not occurred.

SYMPTOMS AND LESIONS

Only two spontaneously infected and several artificially infected (feeding) rabbits were available for a study of the clinical symptoms. After an incubation period of from 2 to 3 days the animals invariably had fever, the temperature being above 40 C., reaching 41.3 C. on one occasion. The hair was rough and shed more readily. In two animals a seropurulent nasal discharge was noticed. Usually they refused to eat, but drank much water. The total loss of weight varied with the individual case but was noted in every instance. There was marked pallor of the mucous membranes due to a distinct drop in the hemoglobin from 90 to 52%. A few animals showed a slight hyperleukocytosis. Every animal developed severe diarrhea preceding death by several days. This symptom was frequently accompanied by a paresis of the posterior extremities and involuntary passage of urine. There was a quickened pulse, labored breathing and general prostration, a drop in temperature and convulsions signalized the approaching termination in from 4 to 8 days after the onset of the symptoms.

The gross lesions were: Emaciation was always more or less marked. The left or right anterior lobe of the lung of the spontaneously infected animals was as a rule covered with a thin layer of fibrin, the lung parenchyma was consolidated in lobar distribution or showed an infarct-like area surrounded by patchy pneumonia. The anterior portion of the left or right middle lobe showed red and gray hepatization. Also the right anterior and heart lobe were similarly affected and the pleural covering of the pericardial sac frequently showed a fine network of fibrin. The bronchi contained a small amount of grayish purulent exudate. The heart muscle was flabby and decidedly fatty. The blood was thin and sometimes of a brownish tinge. The spleen, which was dark brown, engorged, pulpy, and was enlarged, for example, to $8 \times 1.7 \times 1.5$ cm., weighing 6 to 14 gm. (The normal weight averages between 0.45 - 1.1 gm.) Both kidneys were enlarged, yellowish brown in color, petechiated and showed extensive parenchymatous degeneration. The mesenteric lymph nodes were enlarged, soft and juicy, and contained numerous small or large hemorrhages. Not infrequently the hepatic, peri-aortic and iliac lymph nodes were enlarged and distinctly hyperemic. The liver was increased in size and either dark or pale in color, friable and dotted with small necrotic foci of varying sizes. Along the margins large subcapsular brownish-yellow patches were noted. The gallbladder was distended by a dark, olive green, viscid bile; the wall was thin and soft; no changes were visible on the mucous membrane. In the rabbits infected by intravenous injection the gallbladder was leathery, necrotic and the bile thin or thick and purulent.

The bone marrow of the femur was a light yellowish-brown, soft or deep red and showed a few areas suggestive of necrosis. The stomach externally and internally seemed normal. Stringy, bile-tinged mucus filled the duodenum. the mucous membrane was slightly swollen and was sometimes petechiated. All

the Peyer's patches of the jejunum were swollen, deep red, ulcerated or covered by brownish-red scablike sloughs. The contents were greenish and frothy. Sacculus rotundatus was considerably thickened; the lymphatic structure studded with numerous deep hemorrhages and small pinhead-like abscesses. The mucous membrane was covered by a glassy, slightly blood-tinged slime, and in two spontaneously infected animals consisted of extensive cauliflower-like sloughs. In one rabbit the appendix mucous membrane was banded by broad hemorrhagic areas 0.5 cm. in width or dotted irregularly by diphtheric hemorrhagic round or irregular sloughs. A glassy blood-tinged mucus covered the completely or partially necrotized mucous membrane. The extent of these intestinal lesions varied considerably. The artificially fed rabbits (bile method of Besredka) presented a strikingly rigid cecal or colonic wall, together with a diffuse diphtheric necrosis of the mucous membrane in contrast to the scattered ulcerations observed in the spontaneously infected animals.

Rabbits immunized with living organisms or those that had recovered from an acute infection invariably showed pinhead-like whitish or yellowish abscesses in the lymphoid tissues of the sacculus rotundatus or appendix; such findings have been recorded by Theobald Smith⁹ in rabbits infected with *B. suispestifer*. These abscesses were either sterile or contained streptococci. The uterine horns of two rabbits were enlarged, contained partially dissolved putrid fetuses and an endometrium covered with yellowish necrotic areas. In one male rabbit the epididymis were edematous, hemorrhagic and showed on section numerous areas of necrosis. An orchitis was not present. Sexotropic properties apparently characterized the organism of this epidemic.

The histologic examination of some of the organs revealed changes not commonly encountered in paratyphoid infections of laboratory animals. An endarteritis and endophlebitis were noted in every tissue, not only in the spontaneously but also in some of the experimentally infected animals. Again the characteristic hyperplastic reaction regularly found in human typhoid fever was entirely absent or indicated only by a few scattered macrophages and phagocytes. These observations were unfortunately recorded only when our organisms had already lost their original virulence, and additional infection experiments were mostly unsuccessful or resulted in morbid lesions different from those seen in the spontaneous cases. A careful histologic study of paratyphoid infections should be undertaken in order to elucidate the pathogenesis of this group of organisms. Such a study is particularly desirable in the light of the recent reports of Huebschmann,¹⁰ Herxheimer,¹¹ Dawson and Whittington,¹² and others who noted not only bacteriologic but also fundamental anatomic differences between paratyphoid B. and true typhoid infections in man. Our incomplete data are presented in order to encourage such research on spontaneously infected animals when the occasion arises, and they should not be considered an exhaustive inquiry into this phase of the infection.

The pneumonic changes described apparently developed in three of the spontaneously infected animals as a bronchopneumonia with subsequent lobar red and gray hepatization. This associated type of pneumonia developed as a result of pronounced inflammatory processes in a large number of the inter-alveolar arteries and veins. The lumen of these vessels was filled with nests of fragmented and normal leukocytes of varying origin. The endothelium was loosened from the intima and the media and with the adventitia were edematous and invaded by leukocytes. In some vessels fibrin deposits and bacterial plugs

⁹ U. S. Department of Agriculture, Bureau Animal Industry, Bulletin No. 6, 1894, p. 25.

¹⁰ Beitr. z. path. Anat. u. z. allg. Path., 1913, 56, p. 514.

¹¹ Berl. klin. Wchnschr., 1916, 53, p. 648

¹² Quart. Jour. Med., 1916, 9, p. 98.

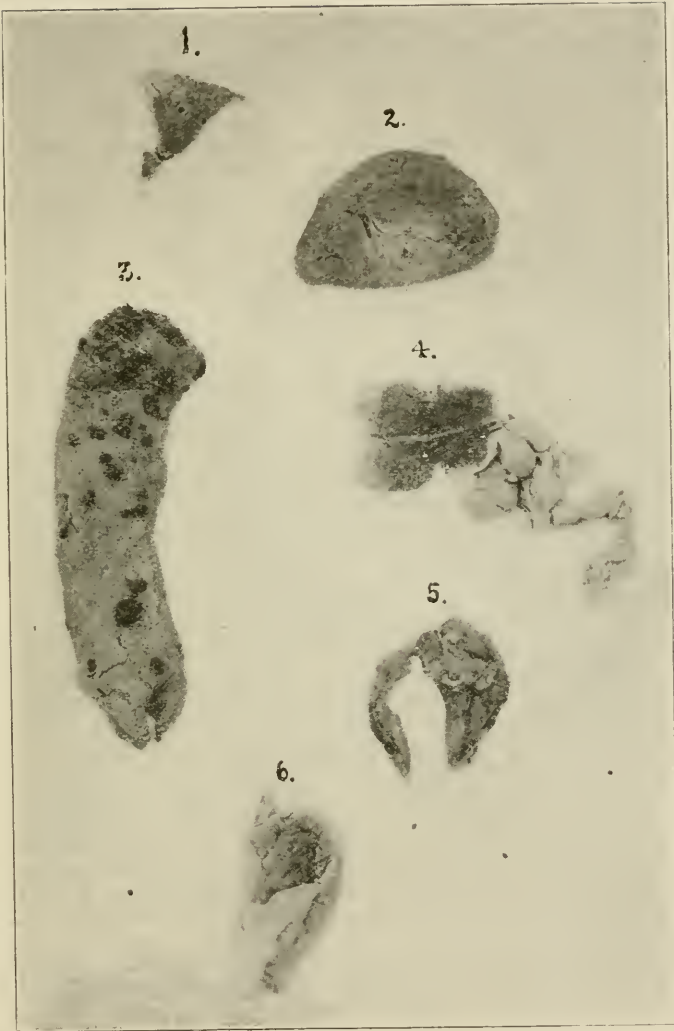


Fig. 1.—Anatomic lesions in rabbit 1371: (1) transverse section of left anterior lobe; (2) kidney; (3) appendix vermiformis opened; (4) mesenteric lymph nodes; (5) incised Peyer's patch; (6) sacculus rotundatus opened, hemorrhagic slough on mucous membrane.

were recognized. Surrounding these inflamed vessels were alveoli containing coagulated albuminous material, fibrin, red cells or leukocytes or even infarcted-like areas. Certain complexes of lobules showed incipient or advanced stages of necrosis, and these areas fused with the bronchopneumonic patches of the lung tissue. The extensive involvement of the terminal bronchi, which were filled with leukocytic plugs extending into the neighboring air cells, suggests that these animals suffered in all probability previous to the paratyphoid infection from the common bronchitis due to *B. cuniculisepticus*. It should, however, be remembered in this connection that Dawson and Whittington, Herxheimer and particularly Klein and Torrey¹³ consider bronchitis and bronchopneumonia as important anatomic lesions in human paratyphoid *B.* infections. Artificially infected rabbits, however, failed to show similar pulmonary changes, which supports the contention of a mixed infection.

The liver sinusoids of all the animals spontaneously or artificially diseased were moderately engorged with blood. The peripheral liver cells contained large fat droplets. Numerous areas consisting of necrosed liver cells and accumulated leukocytes in various stages of fragmentation or typical toxic "pseudo tubercles" were common. In some instances these focal necroses were around the central vein, in others they involved several lobules. Invariably the portal veins or spaces contained thrombotic material and exhibited definite zones of infarction. We have the impression that the necrosis is the end result of a toxin action and not the outcome of embolic blocking of the sinusoids by leukocytes or even splenic cells. Our observations are in this connection quite in accord with those reported by Gruber¹⁴ and Wagner and Emmerich.¹⁵ Bacterial clumps were occasionally seen in the areas of focal necroses. Typical "lymphomas," consisting of macrophages, were not observed. The gallbladder wall was only involved in the intravenously injected animals. The importance of this fact will be discussed below.

The follicles or germ centers of the spleen were usually enlarged and showed a distinct toxic hyaline degeneration, ("eosinophilic hyperplasia") or were completely submerged in the pulp changes. The pulp was engorged with red cells or pigment debris partially inclosed in cells. Numerous necroses, thrombi or fibrin, phagocytic and desquamated pulp cells and nuclear fragments in the splenic sinuses formed a complicated picture. Macrophages were not found, but nests of leukocytes well preserved or partially disintegrated were not uncommon. The cortical sinuses of the mesenteric and portal lymph nodes were slightly distended by leukocytes and hyaline-like debris; the central sinuses, particularly near the hilum, were packed with red cells and large red cell carrying phagocytes and polymorphonuclear leukocytes. No hyperplasia of the follicles or the medullary cords were noted in the large number of nodes examined for this purpose. The spontaneously infected rabbits showed, however, distinct endarteritic changes in several small arteries and patches of more or less marked necrosis in the follicles.

In the jejunum dilatation of the blood vessels and catarrhal hypersecretion of the mucous glands were the most striking changes recorded. The Peyer's patches showed either extensive hemorrhages or complete necrosis of the mucous membrane covering the lymphatic tissue. These changes were accompanied by leukocytic infiltration, at times forming a distinct zone of demarcation. Invariably the submucosal blood or lymph vessels were plugged with leukocytes or thrombotic material. The necrotizing diphtheria inflammatory processes of the sacculus rotundatus, appendix and ileum were confined to the

¹³ Am. Jour. Med. Sci., 1920, 159, p. 546.

¹⁴ Centralbl. f. Bakteriell., O., I, 1916, 79, p. 1.

¹⁵ Ibid., 1916, 77, p. 301.

intestinal wall provided with lymphatic tissue. In these places a confluent mass of necrosis, together with an extensive hemorrhage and little or no fibrin, covered an area of cellular degeneration and disintegration extending to the oedematous muscularis.

The diphtheric changes in the colon of the rabbits infected by first feeding bile and following with the ingestion of large amounts of paratyphoid bacilli resemble those of epidemic dysentery. A phlegmonous infiltration of the entire wall of the mucous membrane was covered by a layer of fibrin and necrotic tissue.

The mucous membrane of the appendix in one rabbit was in its entire distribution necrotic and appeared as a faintly stained mass resting on a circular zone of demarcation just above the lymphatic tissue layer. In some of the immune or recovered animals the follicles of this portion of the appendix contained large areas of necrosis and leukocytic aggregations forming a micro abscess. There were changes in the regional capillaries, but not as marked as those described in the lung and the mesenteric lymph nodes. The lesions are unquestionably different from those reported in human typhoid. They develop in the lymphatic tissues of the intestinal canal but not on the basis of a hyperplastic reaction. Hemorrhages and necroses, apparently the result of vascular injury, predominate and in this respect the anatomic process of "rabbit typhoid" finds an analogy in human paratyphoid or in hog cholera. As far as the appendix is concerned, the lesions remind the observer strongly of those seen in acute human appendicitis; certain stages are strikingly similar to those described by McMeans¹⁶ in his able article on experimental appendicitis.

Some sections of the bone marrow exhibited hemorrhages, leukocytic accumulations and in one instance small scattered areas of necrosis. The myocardium of one rabbit (1415) showed extensive fatty degeneration of the muscle fibers; in the majority of animals indications of a beginning interstitial myocarditis were visible. The blood vessels of the epicardium and myocardium were dilated and frequently possessed nests of leukocytes.

Hyaline thrombi in the glomerular capillaries, hemorrhages, irregular degeneration of the tubular lining, perivascular edema and albuminous tufts were noted in the kidneys. The suprarenals were normal. In one male rabbit the spermatic vein contained thrombotic material and leukocytic debris with adjacent necrosis of a portion of the epididymis. The uterus of two animals contained necrotic embryonic tissue and the endometrium possessed all the signs of a marked inflammation with necrosis. Clinically, as well as anatomically, this spontaneous paratyphoid infection in rabbits differed in no respect from the disease constantly observed and carefully described for this species inoculated with various representatives of the paratyphoid-enteritidis group, by Smith and Moore,¹⁷ Karlinski,¹⁸ Raccuglia,¹⁹ Ordway, Kellert and Husted,²⁰ Okubo,²¹ and others. The descriptions given by these writers cover our observations in every detail as far as the gross morbid lesions are concerned, but we are unable to find the endothelial hyperplasia and the typhoid-like microscopic changes mentioned repeatedly by Ordway and his associates. Vascular changes and necroses predominated in the tissues of our animals. It is not unlikely that the high virulence of the organism and the comparatively short duration

¹⁶ Arch. Int. Med., 1917, 19, p. 709.

¹⁷ U. S. Dept. of Agriculture, Bureau of Animal Industry. Bull. No. 6, 1894, p. 24.

¹⁸ Ztschr. f. Hyg. u. Infektionskrankh., 1898, 28, p. 373.

¹⁹ Arb. a. d. Gebiet d. path. Anat. u. Bakteriologie, 1891-1892, 1. p. 223.

²⁰ Jour. Med. Research, 1913, 28, p. 41.

²¹ Am. Jour. Dis. Children, 1918, 26, p. 376.

of the disease in our rabbits in comparison to the prolonged course of the infection in the observations of Ordway are in part responsible for the differences. However, in the light of recent knowledge on the morbid anatomy of paratyphoid fever in man, the microscopic findings have more than academic interest and urgently deserve additional investigations on suitable material. Such a study appears to us particularly profitable, when we appreciate that the information concerning the factors of pathogenesis, toxigenesis and immunity of the typhoid-paratyphoid group must indeed be considered meager.

THE ORGANISM

Impressions of tissue sections or fragments and direct quantitative plating of the secretions of the spontaneously infected rabbits on brilliant green-eosine-agar or peptic digest endo-agar gave a pure growth of abundant colonies of a gram-negative, motile bacillus. The heart blood of one rabbit (1371) produced 720 colonies per c.c. From the intestinal content, the wall of the jejunum, ileum, sacculus rotundatus and cecum, the liver, the spleen, the lung, etc., a practically pure and profuse growth of the same organism was obtained. The bile did not contain viable bacteria on direct plating of 1 c.c.; on enrichment in broth paratyphoid-like organisms could be demonstrated.

The isolated organisms fermented the following carbohydrates, with the formation of gas: glucose, maltose, levulose, mannite, galactose, dulcitol, mannose, arabinose, sorbitol, rhamnose, and xylose, but had no effect on lactose, saccharose, dextrin, inulin, salicin, raffinose, dextrin and adonite. The fermentation of inositol was slow; acidification was noted only after 48-60 hours' incubation. In lead acetate agar, there was a browning of the medium at the end of 24 hours, and glucose serum water, according to Krumwiede, was reduced. Neutral red glucose agar was split by gas production and reduced. In brom-cresol-purple-milk, the reaction at the end of 24 hours was slightly acid, and remained so for 48-72 hours. After the fourth day the medium turned gradually alkaline and at the end of 20 days was slightly saponified. Gelatin was not liquefied in 30 days and no indol was produced in Difco or Witte's peptone solution. The organisms are highly resistant to brilliant green. The final acidity in glucose diacid phosphate-peptone solution was P_H 4.8-5.0 after 48 hours and P_H 5.0 in mannite after the same period of incubation. The biochemical reactions just mentioned place the organisms isolated with the *B. schottmülleri* type of the colon-typhoid group. The behavior in carbohydrate mediums followed the type III reaction described by Winslow, Kligler and Rothberg in 1917.

AGGLUTINATION

The strains isolated from the various tissues were readily agglutinated in the first generation by a polyvalent human paratyphoid B. (*B. schottmülleri*) serum in a dilution of 1:500 and were not influenced by a highly potent *B. enteritidis* serum in dilutions above 1:10. Subsequent serologic tests were therefore conducted with the representatives of the paratyphoid-suispestifer group. The technique employed in order to classify the rabbit organism was identical to the one recently described by one of us.²²

Specific antisera against paratyphoid B. strains of rodent, avian, porcine and human origin were obtained by subcutaneous injections of living organisms in progressive amounts varying from 0.01 c.c. to 0.2 c.c. of a 24-hour old broth culture at weekly intervals. This mode of immunization produces characteristic local necroses and abscesses.

²² Feusier and Meyer: Jour. Infect. Dis., 1920, 27, p. 185.

According to the chart the bacillus (lung strain 2 and the heart blood strain 1371 behave in an identical manner) is by direct agglutination a paratyphoid *B.* closely related to the rodent type of this large group. It has also been definitely proved that the strain does not belong to either the human, avian (true paratyphoid strains not *B. sanguinarum* or *B. pullorum*) or porcine paratyphosis "B" group. A rabbit antiserum prepared with our organism possessed group agglutinins for the guinea-pig-paratyphoids, for 2 avian and for 2 swine typhus strains. A rabbit and a guinea-pig serum produced with a guinea-pig-paratyphoid strain exhibited group affiliations to our bacillus and to a number of guinea-pig organisms isolated from different epidemics. The striking group agglutination reactions among the avian paratyphoids by these and other serums are noteworthy. A culture isolated by Dr. N. S. Ferry from a rabbit, and sent to us as a representative of the paratyphoids found by him in these animals, behaved more like a member of the avian than of the rodent group. The mutual cross agglutination among the guinea-pig and the avian paratyphoids is strongly suggestive of a close relationship. We are not in possession of the strains employed by Krumwiede, Valentine, and Kohn, and we can therefore not state conclusively that our organism belongs to their group.

Absorption tests failed to classify our organism further in the animal paratyphoid group. Antigenically the rabbit paratyphoid strains are members of the animal paratyphoid group, which can be readily distinguished by absorption tests from the true human *B. paratyphosus*, "B" or *B. schottmülleri* the causative organism of human paratyphoid fever. Further subgrouping of the animal paratyphoid into rodent, avian or porcine groups may be accomplished by direct agglutination, but a more conclusive classification is impossible by the customary absorption technic.

The hog cholera bacillus or *B. cholerae suis* represented in our collection by a number of strains designated as *B. supestifer* and *B. voldagsen* and *B. typhi suis* do not, according to the tests of others and our own, belong to the rabbit group of animal paratyphoids, but are to be classed in a group by themselves. Some paratyphoid strains, of porcine origin, the so-called swine typhus culture I and II are related to our rabbit organisms. These observations support the recently formulated conclusion of Ten Broeck²³ relative to the existence of a distinct variety of paratyphoids common to animals and justify the acceptance of the terminology proposed by him. The organism could be designated as a member of the "*B. aertryckei*-group." Unquestionably further study in this large group of bacteria will, in our opinion, reveal additional subgroups with distinct host relationship.

PATHOGENICITY

The rapid progress of the epidemic obviously suggested a virulent strain of paratyphoid bacilli. As transmission from rabbit to rabbit in all probability resulted by way of the intestinal tract in form of a feeding infection, it was of interest to determine the approximate number of bacteria necessary to cause a fatal issue in these animals. It is generally stated and quite recently emphasized by Besredka²⁴ that the rabbit is nonsusceptible ("*tout à fait invulnérable*") against massive doses of paratyphoid or typhoid bacilli introduced into the digestive tract. This statement deserves some modification. Occasionally recently isolated, parasitic animal and human strains were encountered which can produce typical lesions and generalized infections when administered by

²³ Jour. Exper. Med., 1920, 32, p. 19.

²⁴ Bull. de l'Inst. Pasteur, 1920, 18, p. 123.

CHART I

Direct Agglutination Tests

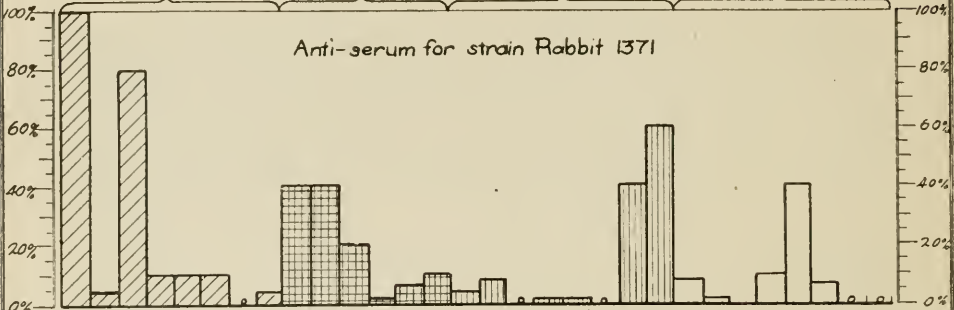
Rodent Origin

Avian Origin

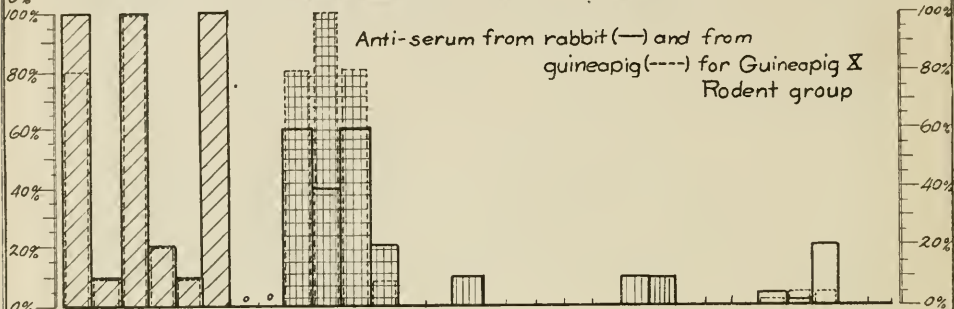
Parvian Origin

Human Origin

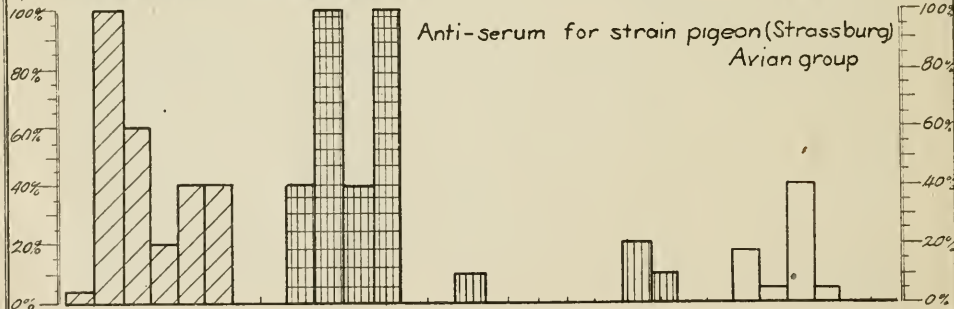
Anti-serum for strain Rabbit 1371



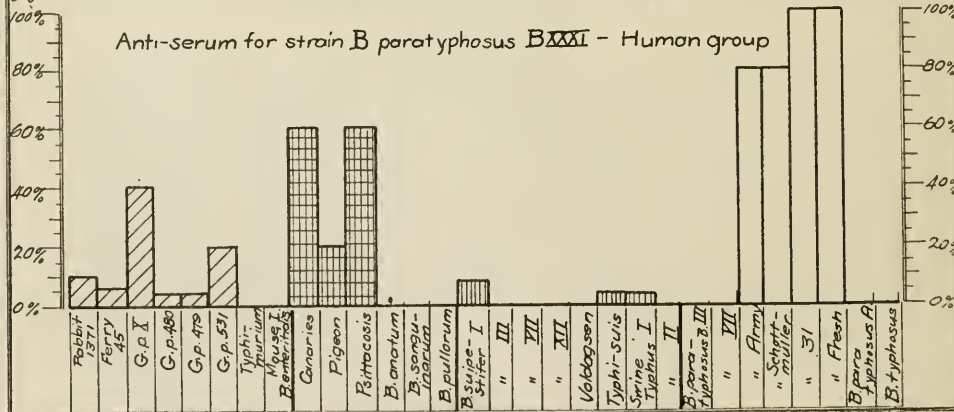
Anti-serum from rabbit (—) and from guineapig (----) for Guineapig X Rodent group



Anti-serum for strain pigeon (Strassburg) Avian group



Anti-serum for strain B paratyphusus BXXXI - Human group



mouth. From a comparative standpoint it was also important to test our parasitic and saprophytized organism on other laboratory animals, particularly mice and guinea-pigs.

Inoculation Experiments.—Mice and guinea-pigs inoculated subcutaneously with 0.1-0.01 cc of a 24-hour broth culture succumbed in from 12 to 24 hours. The inoculated organism was found in the heart blood and in the organs. Even 15 months after isolation the strain retained approximately the same degree of virulence.

Subcutaneous inoculations of 0.1-0.01 cc of a 24-hour broth culture 2½ months after isolation produced in a large number of rabbits more or less extensive necroses and local abscesses. A rise in temperature for several days, loss of appetite, perhaps temporary diarrhea and an enlargement of the regional lymph nodes accompanied the local processes. Ten days after the inoculation the blood serum of such rabbits agglutinated the paratyphoid bacillus in dilutions from 1:400-1:800. Two additional injections produced local lesions only and usually immunized the animals completely as will be shown in another paper. Judging from the observations of Ten Broeck²⁵ with *B. suis*, our bacillus possesses in comparison a low virulence on subcutaneous application and our findings lend little support to the supposition that fleas or other insects could be considered as factors in the epidemic.

The intravenous inoculation, on the other hand, was fatal in comparatively small doses in from 2-10 days. The number of recently isolated cultures necessary to kill adult rabbits of different litters and age varied between 200,000 and 700,000 organisms in broth cultures of different compositions and reactions. The six month old strain was pathogenic in doses of 1-2 billion and after one year this number of bacteria was fatal only in young rabbits. Irrespective of the variation in virulence the clinical symptoms, following a distinct incubation time and the anatomic lesions of a septicemia, were practically the same. Of particular interest are the findings of a diphtheric or even necrotizing cholecystitis with positive cultural results in every nonimmunized rabbit which succumbed to the intravenous injection.

These experiments only demonstrate that the rodent paratyphoid bacillus possessed for laboratory animals an average virulence, which differed in no respect from the one ordinarily noted with recently isolated human or animal paratyphoid strains.

Feeding Experiments.—In the feeding experiments on coccidiosis-free rabbits the cultures were grown on agar slants and in peptic digest broth. Only cultures 24 hours old were used. The data obtained indicates that the ingestion of 2 billion recently isolated organisms produced the disease with characteristic lesions. When the strain was 6 months old the results became irregular with amounts as high as 8 billion, and when 12 months old even 1,600 billion were innocuous in a large series of animals. At the time of writing enormous doses (5,000 billion in milk) are necessary to produce an infection by feeding. Even 20-50 billion organisms are required to confirm the observations of Besredka²⁶ of feeding bile preceding the administration of organisms of the dysentery-paratyphoid group. The virulence of the organism has progressively degenerated and infected rabbits shedding these paratyphoid organisms can with impunity be mixed with susceptible ones. On the other hand, the epidemiologic data presented indicated a high original virulence which unquestionably

²⁵ Jour. Exper. Med., 1918, 28, p. 759.

²⁶ Ann. de l'Inst. Pasteur, 1919, 33, p. 557.

increased by passage from animal to animal, probably in a manner somewhat similar to the one described by Ten Broeck.²⁷

Even guinea-pigs were endangered, though spontaneous cases were not observed by us. Ingestion of 250,000 organisms was fatal for guinea-pigs varying in size from 400-500 gm. in from 4-8 days, using the recently isolated culture. The necropsy findings differed in no respect from those commonly noted in rodent paratyphoid or pseudotuberculosis. The virulence of cultures 6 to 12 months old diminished similarly for guinea-pigs as for rabbits, and larger doses were necessary to produce an infection. Daily feeding of a whole growth of an agar slant (culture 18 months old) on minced carrots for 3 weeks produced a chronic disease with positive cultural findings in the spleen, mesenteric lymph nodes and liver. It was noted that white mice that accidentally had access to the remains of the carrot mixture succumbed to an acute infection (10-12 days), with typical lesions and positive cultural findings in all organs. At least for this species of animal the organism had retained a considerable degree of virulence.

The feeding experiments on rabbits furnished a series of observations that appear to be of considerable importance and therefore deserve some consideration. Contrary to our findings in the intravenously inoculated rabbits in which a diphtheric or necrotized cholecystitis with positive paratyphoid bacilli findings were recorded, the rabbits fed and successfully infected with the same organism of the same age did not show a cholecystitis. Invariably the cultures obtained from the bile were sterile. These results are fully in accord with those described for the spontaneous cases. We also recall in this connection that the recent statistics of Hübener²⁸ and Herxheimer¹¹ on acute paratyphoid fever infections in man fail to mention pathologic changes in the gallbladder. One is probably justified in assuming that in their cases, at least, this organ was not diseased. These facts may contribute some information concerning the pathogenesis of cholecystitis due to organisms of the typhoid-paratyphoid group in rabbits free from coccidiosis or other infections of the liver. One fact stands out preeminently, namely, irrespective of the number of paratyphoid bacilli in the blood and tissues at the time of death, the animals that received their infection by way of the intestinal tract did not show a cholecystitis nor even bacilli in the bile so perfectly suited for their proliferation. The gallbladder wall may on enrichment in broth be found to be infected, which is not surprising when the blood stream is teeming with specific organisms.

It will be the purpose of another series of papers to discuss in detail the manner in which typhoid bacilli, for example, reach the gallbladder and bile in rabbits, but we can already state that an ascending invasion via the common and cystic duct has not been observed in our studies. On the other hand, there is conclusive evidence at our disposal which indicates that the infection is descending. Typhoid or paratyphoid bacilli inoculated intravenously in certain doses, which vary according to the virulence of the organisms, the size of the animal and other factors to be considered elsewhere, appear in the common duct bile in the first 10-15 minutes after the injection. The bacteria reach the bile by way of the biliary passages and proliferation in this secretion or the constant feeding of the same from liver foci will lead to a more or less prolonged sojourn of the organisms with secondary inflammatory involvement of the gallbladder wall. In our experience another route of bile infection is occa-

²⁷ Jour. Exper. Med., 1917, 26, p. 437.

²⁸ Fleischvergiftungen und Paratyphus infektionen, 1910, p. 118.

sionally noted in rabbits, namely, the embolic invasion of the capillaries of the gallbladder wall. This mechanism of invasion is particularly common when virulent strains in large doses are used. The histologic picture of the gallbladder wall of the intravenously inoculated rabbits in our pathogenicity series suggested such a hematogenous infection as the most likely mode leading to the diphtheric or necrotized cholecystitis. Bacterial thrombi are found in the capillaries at the base of the fundus of the gallbladder mucous membrane. These foci are surrounded by areas of necrosis or leukocytic infiltration. Such portions of the mucosa may be covered by an intact epithelium. In the majority of cases, however, the complete destruction of the entire mucous membrane, muscularis and serosa does not permit an insight into the cycle of events leading to the complete destruction of the gallbladder. The difference in the gallbladder lesions between the spontaneously infected, the experimentally fed and the intravenously inoculated rabbits may therefore be readily explained as follows: Paratyphoid bacilli intravenously introduced rapidly accumulate in large numbers in the liver capillaries; the gallbladder as an appendix of this organ and connected by accessory portal veins (Violle²⁹), receives a proportional share in its small blood vessels. In the parenchyma of the liver the characteristic necroses and cellular proliferation develop as a result of the multiplication of the retained bacteria; in the gallbladder wall a similar process leads to a diphtheric necrosis of the mucous membrane with subsequent invasion of the bile. The latter may also receive bacteria simultaneously from the biliary passages. Intensive proliferation in this secretion leads to additional destruction of the mucous membrane from within and in turn may cause complete necrosis, even beginning perforation of the gallbladder wall. In the spontaneously infected or in the fed animals the number of organisms that reach the liver is comparatively small and the invasion gradual. The protective properties of the liver (also seen in immunized rabbits inoculated intravenously) prevents the indirect passage of paratyphoid bacilli from the hepatic veins to the biliary capillaries. A few bacteria reaching the bile are probably rapidly discharged in the hepatic duct bile, which is continuously flowing as result of the toxic enteritis, and in all probability no organisms reach the cystic bile where multiplication is most suitable. This reconstruction of the events is borne out by the cultural studies in this and other series of animals studied for the same purpose. The gallbladder bile, the duodenal and jejunal contents were free from paratyphoid bacilli. Such bacilli were found only around or below the diphtheric or ulcerated agminated follicles and in the region of the sacculus rotundatus and appendix. On the other hand, the gallbladder wall of the fed or spontaneously infected animals was found to contain a few paratyphoid bacilli; this is not surprising when we realize that on one occasion 720 organisms were present in 1 c.c. of heart blood. A direct, heavy invasion of the gallbladder wall and its blood vessels was made impossible on account of the gradual infection of the liver from the portal system. Consequently, the formation of bacterial emboli primarily responsible for the diphtheric inflammation was eliminated. These observations furthermore show that in the rabbit at least a portal septicemia with rabbit pathogenic virulent and invasive paratyphoid bacilli does not necessarily lead to a cholecystitis or to an infection of the bile. On the other hand, the intravenous injection of the same organism in comparatively small numbers causes a hematogenous invasion of the gallbladder and bile by way of the biliary passages on one hand and by way of the capillary thrombi in

²⁹ Ann. de l'Institut. Pasteur. 1912, 26, p. 384.

the wall on the other. In case the animal has lesions of coccidiosis or the organism possesses specific selective organotropic properties for the gallbladder, as was shown by Fraenkel and Much,³⁰ localization in this organ may occur following any method of infection. These facts have in our opinion a bearing on the typhoid-paratyphoid carrier problem in laboratory animals, which we will discuss elsewhere more in detail.

TOXIN PRODUCTION

Smith and Ten Broeck³¹ observed that bacteria of the typhoid-paratyphoid group are capable of producing highly potent toxins for rabbits in peptonized sugar-free veal broth plus 0.1% glucose incubated in shallow layers. One of us (K. F. M.) several years ago conducted a large number of experiments on dogs and rabbits with the toxic filtrates from *B. abortus equinus* prepared according to the procedure given by these writers. The results were rather irregular and at that time it was impossible to investigate the factors responsible for the inconstant results. A series of tests conducted with the paratyphoid bacilli isolated from the rabbit apparently offer an explanation for the failures recorded. It was found that aside from the depth of the layer of fluid in which the cultures were grown, namely, not more than 2 cm., the reaction of the medium is an important factor. We originally prepared the substratums for the production of toxic filtrates strictly according to the formula of Smith and Ten Broeck with Witte's peptone, adjusting the fluid to an initial reaction of 1% acid to phenolphthalein. The final product had a H-ion concentration of P_H 7.2-7.4. Several batches of mediums with this reaction were tried. Invariably the toxic filtrates of 7-day old cultures, inoculated in doses of 1 c.c. per kilogram of weight, produced temporary restlessness, labored breathing followed by slight stupor, passage of urine and feces and loss of weight of from 75-100 gm., but never death. Experiments with other toxins and particularly the generally known fact that diphtheria toxin is only produced at a P_H of from 7.8-8.4 suggested some experiments with mediums adjusted to such a reaction. Powerful toxic filtrates, even from 48-hour cultures, were obtained in a broth with a P_H of 8.4. For illustration of this statement, we cite in detail one experiment:

Rabbit 1749, weighing 2,350 gm., received at 2:30 p. m., 2.5 c.c. of filtrate of a 72-hour old broth culture of paratyphoid bacilli 1371 (initial reaction of medium P_H 8.4) into an ear vein. At 4:30 was drowsy and breathing labored with a snuffling noise, hind leg extended, eyes dull and partially closed. At 9 p. m. the rabbit was found dead. The necropsy showed hemorrhages in the abdominal and pelvic lymph nodes, engorgement of the liver, and spleen, petechiae on the serosa and mucous membrane of the stomach and the duodenum. There were 2 c.c. of fluid in each pleural sac; the lung was slightly congested and moist; there were intense congestion in the trachea and petechiae on the pericardium; the blood was thick and tarry.

The course of the intoxication and the necropsy findings were practically identical in all the animals injected with these toxins and in a general way differed in no respect from the description given by Smith and Ten Broeck and Mulsow³² for various representatives of the typhoid-paratyphoid group.

In connection with some other problems, we were interested in the possibility of producing toxic organ filtrates according to the procedure of J. T.

³¹ Jour. Med. Research, 1914, 31, p. 523.

³² Jour. Infect. Dis., 1919, 25, p. 135.

Parker.³³ This writer demonstrated that the liver of rabbits inoculated with cultures of *B. typhosus* or *B. prodigiosus* under certain conditions contain a toxic substance extractable with salt solution. When the toxic extracts are injected intravenously into normal rabbits these animals develop symptoms resembling those of anaphylactic shock and succumb. We have confirmed these observations with the *B. typhosus*, but thus far we have been unable to obtain toxic liver extracts of rabbits injected with one half to one slant of our paratyphoid bacillus. Even by producing the most favorable conditions by rendering the animal very sick in 6-10 hours after the injection of one half slant of a young culture no liver poison was elaborated that would kill small rabbits in the chosen dose of 10 cc of extract. Our paratyphoid bacillus does, however, behave in a similar manner to a number of other paratyphoid strains tested by the same technic. Certain immunity tests to be reported elsewhere suggest that the toxemic manifestations of paratyphoid infections in rabbits are different from those constantly observed in these animals injected with typhoid bacilli. A progressive septicemia with a high bacterial count of the blood and tissues precedes the death of paratyphoid rabbits. On the other hand, the fatal issue in rabbits injected with typhoid bacilli is frequently accompanied by a comparatively low bacterial blood and tissue count. It is not unlikely that the greater bactericidal power of the blood and tissues of the rabbit for typhoid bacilli is responsible for the rapid destruction of viable bacteria and the production of a large amount of poison which causes the death of the animals. These bacteriolytic forces are apparently not operative to the same degree in paratyphoid infection in rabbits, and poisons are not elaborated in the tissues; or the liver is able to neutralize large amounts of toxin as they are formed, and a quantity which exceeds the detoxicating threshold is never produced. In both instances, the poison naturally cannot be demonstrated in the salt extracts. Whether these interpretations are the correct ones or whether they only represent one phase of a more complex mechanism is at present the subject of a more extensive investigation.

GENERAL SUMMARY

The foregoing study demonstrates the occurrence of spontaneous paratyphoid *B.* infections in rabbits. Unfortunately it was impossible to determine the origin of the disease; the dealer supplying the infected rabbit, which initiated the epidemic described, had disposed of his stock when the nature of the malady was properly established. It would have been of considerable practical importance to have known whether the existence of carriers was responsible for the development of the acute cases. The possibility that certain types of abortion in rabbits may be caused by paratyphoid bacilli and that animals in such a condition may disseminate the infection cannot be dismissed in the light of our observations. It must also be the question to be decided whether or not the causative strain was of guinea-pig or of mouse origin. The fact that the dealer did not raise guinea-pigs or mice and the extreme rarity of paratyphoid *B.* infections in rabbits in

³³ Jour. Exper. Med., 1918, 28, p. 571.

contrast to the rather ubiquitous distribution of this malady in guinea-pigs and mice, does not lend much support to this conception. Rabbits are frequently exposed to pseudotuberculosis of guinea-pigs. Repeatedly diseased animals have been introduced into our cages, and before the infection was recognized, numerous rabbits have been exposed to their discharges without causing a paratyphoid epidemic. Moreover, the agglutination tests conducted with the strains isolated from a series of different guinea-pig epidemics in our laboratory failed to indicate a close relationship to the rabbit-paratyphoid organism. The determinations of the source of paratyphoid infections must therefore be reserved for future investigations.

Our observation, which shows how readily an epidemic among laboratory rabbits may get started, should be a lesson to all laboratory workers. It is obvious that careful necropsy and complete cultural examination should be performed on all stock animals, even if the circumstances of their death suggest only an accidental infection. It has been our policy to keep complete records of the origin of the animals received and to place the same in experiments only after a strict quarantine in isolated cages for at least two weeks.

This discussion cannot be closed without a brief consideration of the relationship of our paratyphoid organism to so-called food poisoning. Although MacConkey⁸ reports cases of food poisoning in which rabbit meat was involved, it is definitely shown by the serologic tests that our bacillus does not antigenically belong in the human paratyphoid or *B. schottmülleri* group. Yet the ability of this organism to evolve a potent toxin should be considered in any attempt to explain outbreaks of food poisoning in which rabbit flesh is a part of the suspected meal. Even if the reports on meat poisoning caused by rabbit meat are limited to the one by MacConkey, it would be most unwise to say that this food cannot cause disease in man and to use this as an argument for the safety of man against the animal paratyphoid bacilli. Individual susceptibility may play an important rôle and a nonpathogenic organism may be readily transformed into a potent factor of gastro-enteritis.

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A Hemophilic, Anaerogenic Paracolon Bacillus
Found in a Case of Infected Bilat-
eral Hydronephrosis

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A HEMOPHILIC, ANAEROGENIC PARACOLON BACILLUS FOUND IN A CASE OF INFECTED BILATERAL HYDRONEPHROSIS

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In the course of a systematic investigation of urinary infections primarily undertaken with the aim to reclassify by modern methods the various causative types and the subdivisions of the colon-paracolon group, we found an organism which was only cultivated on hemoglobin medium. This bacillus reverted atavistically to a saprophytic nongas-producing paracolon after artificial cultivation. A similar transformation of type took place in the urine of the host following vaccination and clinical recrudescence. The organism is here described and an account given of the case from which it was obtained.

HISTORY OF THE CASE

A woman, aged 35, had always enjoyed excellent health up to a short time following her last childbirth. May, 1915. There was no history of focal infection such as teeth abscesses or tonsillitis. Following childbirth she had intermittent attacks of chills and fever which were attributed to an infection in the broad ligament of the left side. The urine was negative on examination and cultural study was not made. Later in May examinations of the urine showed a bacilluria due to a peculiar type of gram-negative (colon bacillus?) bacillus with a few polynuclear cells and a few hyaline casts. A blood culture was negative; spleen large and soft; marked hemorrhoids, not infected. The general poor condition continued; the hemorrhoids were troublesome and she complained of pain in the neck, principally in the back, and asthenia. In August, 1917, the urine still showed a slight trace of albumin with a number of white cells and bacteria. The patient was treated by rest in bed, regulation of diet, and control of the bowels in an attempt to relieve the urinary infection. In the latter part of 1917, the hemorrhoids were removed by operation. Complete gastro-intestinal studies made about this time were negative with respect to gallbladder or intestinal focus.

In June when the patient first came under our observation, the total phthalein output was 65% and bacteriologic study of the urine showed a gram-negative bacillus which grows only on blood agar plates. A stool specimen examined in June showed normal flora. Plain roentgenograms of kidneys, ureters and bladder were negative. A more complete urologic study later in June showed a condition of bilateral hydronephrosis with a tortuous ureter and movable kidney on the right side. On the left the hydronephrosis was apparently due to a valvelike obstruction at the ureteropelvic juncture. Infrequent attacks of fever with dull

pain in the right or left side and severe headaches lasting for several days continued to recur. The patient was vaccinated during the months of July, August and beginning of September with a heat-killed suspension of the hemophilic organism and *B. alkaligenes*.

In Sept., 1918, ureteropyelography confirmed the previous finding and showed a rather dilated and tortuous upper right ureter and a hydronephrosis with a pelvic capacity of 40 cc and marked blunting of the minor calyces. The pyelogram of the left kidney was definitely square-shaped but its ureter was not dilated. The left pelvis had a capacity of approximately 45 cc. The separate phthalein output showed marked variation according to whether the tip of the ureteral catheter had entered the respective pelvis or not, as for example, on March 11, 1919, phthalein with the right catheter not in the right pelvis, but the left one well up in the pelvis appeared on the left side in 5 minutes, right side 8 minutes; the first 15 minute output, right side 2%, left side 15%, whereas, on March 14, with the position of the catheters reversed, the phthalein output on the right was 10% and on the left 5%. Total phthalein on March 13, 1919, was 30% first hour—second hour not taken. On April 10, total output was 27% first hour, and 11% second hour. Repeated phthalein estimations have shown a very definite and progressive diminution in renal function during the last 2 or 3 years.

The bacteriologic results with catheterized urine specimens obtained by us from June, 1918, to April, 1919, follow:

June 6, 1918: Bladder Urine: hemophilic organism, streptococci.

June 8, 1918: Bladder: hemophilic bacillus and *B. alkaligenes*. Right Kidney Urine: sterile, direct and enriched. Left Kidney Urine: hemophilic bacillus; enriched in plain broth sterile.

June 10, 1918: Bladder Urine: hemophilic bacillus and *B. alkaligenes*.

June 14, 1918: Bladder: hemophilic bacillus. Right Kidney: sterile direct; enriched in blood broth; hemophilic organism. Left Kidney: hemophilic bacillus.

June 18, 1918: Bladder: hemophilic bacillus. Right Kidney: sterile direct, enriched few cocci. Left Kidney: hemophilic bacillus and *B. alkaligenes*.

Sept. 15, 1918: Bladder direct: ∞ anaerogenic paracolon growing on non-hemoglobin mediums and *B. alkaligenes* in one drop of urine. Right Side: *B. alkaligenes*. Left Side: sterile, direct and enriched.

Sept. 19, 1918: Bladder: ∞ paracolon few *B. alkaligenes*. Right Side: few paracolon and innumerable *B. alkaligenes*.

March 11, 1919: Bladder: ∞ paracolon. Right Side: sterile. Left Side: ∞ paracolon.

April 26, 1919, a plastic operation on the left renal pelvis was made under gas and oxygen—Heineke-Mikulicz incision being made at the ureteropelvic juncture to enlarge the pelvic outlet. Culture of the urines obtained May 16 showed an entirely different type of infecting organism, namely, a pure colon bacillus, the typical organism previously found having entirely disappeared. The patient has been under observation since the operation to the present time and there has been no reappearance of the previous type of infection.

BACTERIOLOGIC FINDINGS. THE HEMOPHILIC ANAEROGENIC PARACOLON BACILLUS

The first specimen of catheterized urine collected in June, 1918, was plated in routine fashion on endo and sheep blood-veal agar plates; 4 cc of clear urine were also enriched in plain glucose broth. After 24 hours' incubation only 3-4 small, slightly reddish streptococcus colonies were observed on the endo-plates; the blood medium, however, was covered with innumerable discrete whitish

colonies not unlike those of *B. influenzae*. On closer inspection the isolated colonies were somewhat raised and very viscid on touch with a platinum needle. Microscopically, the growth consisted of short, stumpy or coccoid gram-negative bacilli. The enriched broth tubes were clear and showed only a faint, filmy sediment. Veal, peptic digest, casein and veal-ascitic fluid—or beef serum-agar plates with and without blood gave an identical result, namely, small colonies appeared only on the blood plates. Even after incubation for from 5-10 days no growth was visible. The phenomenon repeated itself, in the course of frequent cultures made from the urine of the bladder and ureters; as a rule the *B. alkaligenes* grew on all the mediums employed yet the predominant bacillus was only cultivated on a hemoglobin containing substrate.

Specimens of urine obtained on Sept. 15, 16, 19 and thereafter until March 11, 1919, gave, however, a very fine dewdrop-like growth of the originally strictly hemophilic organisms on ordinary plain or glucose or serum agar after 48-72 hours' incubation. On blood plates the growth was more profuse, the colonies were larger and somewhat darker in color. In the meantime, the strictly hemophilic strains having been kept on blood agar and tested at weekly intervals on plain agar, had acquired the property to grow fairly easily on blood-free mediums. On the average 10-12 transplantations on blood mediums were necessary to convert the parasitic, hemophilic type into a saprophytic one, which as such permitted a definite classification.

For the sake of clearness it appears advisable to describe collectively the findings on the parasitic strains isolated from June, 1918, until March, 1919, and those of the saprophytic strains as they developed either in the fresh specimen since Sept., 1918, or in the test tubes as a result of frequent transplantations. In this connection it may be stated that only purified cultures were investigated and that on several occasions 50 colonies of the hemophilic organism were tested on plain agar slants. Detailed studies were, however, made only with 5 representative offsprings of the parasitic and of the saprophytic colonies, respectively. The composition and reaction of the mediums were always identical and the differences between the two types of the same organism can therefore only be explained on the basis of an adaptation phenomenon. The characteristics of the strains are:

1. *Parasitic Strains*: Morphology: The urinary sediment of the left ureter as a rule gave a pure culture of the organism under consideration. Smears showed small coccoid-like bacilli which in shape and size resembled *B. melitensis* or certain forms of *B. pseudo-influenzae*; they were always immotile. In stained preparations they appeared as gram-negative short rods (0.3-1.5 mikrons), usually arranged in clusters. Some forms may show indications of a capsule. Material from cultures emulsified poorly but stained readily; the single organisms were generally in the first generation surrounded by a halo, suggesting a zooglea-like capsule, which was easily demonstrated tinctorially by treating the fixed smear with weak acetic acid. Some forms stained bipolarly and resembled in size *B. coli*. No flagella could be made visible. In old liquid cultures long filaments and other pleomorphic forms were constantly noted. The single organisms always appeared separated by a mucoid-intercellular substance.

Cultural Characteristics.—On blood plates small gray-whitish colonies developed readily inside of 24-36 hours under aerobic or semi-anaerobic environment. The bacillus was mesophile, the optimum temperature for growth was 37 C. At 22-25 C. occasionally a poor and slow growth was noticed. Under 20 C. no growth took place. In the course of a few days the colonies appeared

rather raised, conical, slimy and moist; they were very viscid and of mucous consistence. Occasionally threads of from 2.5 cm. in length could be easily withdrawn by touching them with a needle. The medium was not altered in color; there was no hemolysis. On blood plates and particularly on cooked hemoglobin mediums, a film-like, diffuse growth with a slight brownish discoloration in the butt was readily obtained. The water of condensation showed a stringy, slimy sediment. In blood broth a whitish sticky film covered the layer of red cells; after 6-8 days' incubation the hemoglobin was discolored and perhaps a slight turbidity of the supernatant broth was visible. The later reaction, being in our opinion the result of acid-split products, was particularly noted in glucose-blood broth tubes. Repeated attempts to grow the bacillus on various other hemoglobin free mediums failed. In sterile urine or urine broth no growth was obtained. Successive transplantations on blood agar produced a profuse saprophytic growth, which after 10-12 series was successively transferred to plain and to glycerin agar. The viability even on blood plants was only slight and weekly transfers to fresh mediums were necessary to keep the organism alive. Heating from 53-54 C. for 30 minutes sufficed to kill the organism when heavily suspended in salt solution.

2. *Saprophytic Strains*.—Morphology: Tinctorially and otherwise the strains differed in no respect from the hemophilic ones. When first isolated they fully possessed a distinct capsule which was, however, lost by artificial cultivation on plain agar; it was retained for about 10-20 generations on glycerol agar or broth.

Cultural Characteristics.—On peptic digest or glycerol or glucose agar plates seeded with urine obtained after the patient had been vaccinated with a suspension of the hemophilic bacilli, very small, streptococcus-like colonies made their appearance as a rule after 36-48 hours' incubation. In the course of 5-10 days these colonies increased slightly, acquiring a more slimy and raised appearance. The margins were sharp or slightly irregular. On glycerol agar the colonies were somewhat larger, markedly convex and very slimy; when touched with a wire loop the entire colonies were usually removed. Even on slants there was no tendency for spreading. The water of condensation had a slimy sediment. Broth cultures (glucose and glycerol) inoculated with the second or third generation showed occasionally a faint turbidity after 24 hours' incubation, which gradually disappeared in the course of another 24 hours; the tubes showed a clear upper part with a tenacious, slimy sediment. By shaking, a tuftlike formation was obtained, which remained for some time in the glycerol tubes the whole medium being very viscid. There was no gas, but a slight acid production in glucose broth. On Loeffler's serum and ascitic fluid agar a fairly thick, more or less compact viscid deposit was formed. On potatoes the recently isolated saprophytic strains failed to grow; the older strains cultivated artificially for over one year and six months gave a faintly visible film. Milk was not coagulated, but slightly acidified after from 4-6 days' incubation. Gelatin plate or stab cultures show after from 10-12 days small punctiform colonies with a finely granular inside structure. This medium is never liquefied, strains grown on glycerol or plain agar become more and more saprophytic and the cultures on the hand to day grow quite freely, but in comparison with *B. coli* less abundantly. Two strains under observation exhibit in this respect growth characteristics which resemble those of a stock culture of *B. dysenteriae* Shiga. In liquid mediums and in brain suspensions the bacteria will remain viable for at least 2 to 3 weeks.

Some of the nonhemophilic strains produced indol in the second and third generation. Highly saprophytized strains have failed to give this reaction in

various peptones (Witte's, Difco, Parke, Davis and Co.). Lead acetate and neutral red remained unchanged; the methyl red test was positive, the V/P reaction was negative. The strains tested quite recently were more brilliant green tolerant than *B. coli* or *B. dysenteriae*. Based on the above cited characteristics the group number of our bacillus is B. 222.2332033.

The fermentation reactions, namely, production of acid, noted in Hiss serum water or peptone-phosphate solutions, are summarized in table 1. For comparison two anaerogenic colon strains, also isolated from urinary infections, are included.

TABLE I
THE CULTURAL CHARACTERISTIC OF ANAEROGENIC STRAINS ISOLATED FROM
URINARY INFECTIONS

	Hemophilic Anaerogenic Paracolon	Anaerogenic Metacolon	Anaerogenic <i>B. coli</i>
Motility.....	0	0	±
Gelatin liquefaction.....	0	0	0
Milk.....	Alkaline or acid P _H 6.6 (10 days)	Alkaline	Acid-coagulated (5 days)
Indol.....	±	±	+
Lead acetate.....	0	0 or +	0
Neutral red.....	0	0 or +	+
Glucose.....	A. P _H 5.5 (5 days)	A. P _H 5.8	A. P _H 4.8
Levulose.....	A.	A. P _H 5.5	A.
Galactose.....	A.	A.	A.
Mannose.....	A.	—	A.
Mannitol.....	A. P _H 5.8 (5 days)	0	A. P _H 4.8 (5 days)
Maltose.....	A.	0	A.
Rhamnose.....	A.	0	0
Xylose.....	A.	0	A.
Arabinose.....	A.	0	A.
Sorbitol.....	A.	0	A.
Dulcitol.....	0	0	A. and 0
Adonitol.....	0	0	0
Lactose.....	0	0	A.
Sucrose.....	0	0	0
Raffinose.....	0	0	0
Erythrite.....	0	0	0
Salicin.....	0	0	A.
Dextrin.....	0	0 or A. slight	0
Inulin.....	0	0	0
Inosite.....	0	0	0
Glycerin.....	0	—	—
Voges-Proskauer.....	0	0	0
Methyl red.....	+	+	+
Urine.....	Alkaline (15)	Alkaline	Acid

In this connection we emphasize the fact that only saprophytic strains, after ten successive transplantations on plain agar were tested, the exact nature of the organism being recognized only at a period when all the original hemophilic strains had acquired saprophytic properties. The tests were repeated recently with the strains kept under artificial cultivation for over a year and results identical with those noted September, 1918, and August, 1919, were obtained. Acid, but no gas is formed by our bacillus in mediums containing the various hexoses, mannitol maltose, rhamnose, xylose, arabinose and sorbitol, but not in dulcitol, adonitol, sucrose, raffinose, salicin, dextrin, inulin, inosite and

glycerin. The acid fermentation is rather sluggish and the end reaction never goes below $\text{pH } 5.5$. These characteristics would place our organism with group III of the classification of Winslow, Kligler and Rothberg; some reactions, however, indicate a relation to group IV.

Agglutination Tests.—The cultural characteristics cited above, suggested the agglutination reactions to place this organism more satisfactorily in the dysentery-paratyphoid group. It was immediately realized that such a procedure would be of limited value on account of the capsulated nature of the bacteria. The negative results obtained with a variety of specific serums testing the capsulated parasitic and saprophytic strains, are therefore of no significance. Even the serum of a rabbit highly immunized with such strains failed to agglutinate the immunizing bacillus. As already stated, the parasitic strains gradually lose their capsules and it is with such organisms that a new series of agglutination tests was set up. Again, negative results were recorded. A rabbit-immune serum with a titer of 1:400 agglutinated only several strains of our bacillus. A large series of anaerogenic paracolon and colon strains were clumped in dilution of 1:2 or 1:5. The same strains were, however, also agglutinated by normal rabbit serums in the same dilutions.

The negative agglutination test obtained with the patient's serum must be ascribed to the use of a capsulated organism and cannot serve as a criterion for the nonpathogenicity of this paracolon bacillus. With the saprophytic noncapsulated strains additional tests were only possible at a time when the urinary flora had been displaced by a typical *B. coli* communior. In this connection we desire to call attention to the importance of serologic studies in urinary infections. It is not uncommon to find negative agglutination reactions¹ in chronic pyelitis and cystitis, and even after a prolonged, intensive vaccination that may result in complete recovery such tests can be entirely negative. Observations of this character have more than academic interest and should therefore be investigated in detail.

Pathogenicity.—Young guinea-pigs and mice inoculated intraperitoneally with $\frac{1}{10}$ slant of a blood-agar culture of the parasitic strain may die in from 24-48 hours showing at necropsy a muco-fibrino-purulent peritonitis. Subcutaneous injections produce a slight infiltration. Rabbits on intravenous application tolerate one-half and even one slant of the same organism. The saprophytic strains are only fatal for guinea-pigs in 1-2 slant doses. Other animals were not used for pathogenicity tests. A specific localization in the urinary passages of rabbits was not noted in a small series of experiments. Old cultures contain apparently toxic substances, which produce on intravenous inoculation of rabbits transitory illness; rhinitis, rapid respiration and diarrhea are usually observed for from 10 to 12 hours.

Intravesicular injections of rabbits with parasitic and saprophytic strains failed to produce a cystitis; the introduced organism could be demonstrated for from 24-72 hours.

B. alkaligenes.—The concomitant *B. alkaligenes* regularly found in the urine from the bladder or from the left or the right ureter corresponded in every respect with the type strain at our disposal. Culturally the colonies of this organism were characteristic and could be distinguished from the anaerogenic, hemophilic paracoli. Transformation of properties, which could suggest a close relation with these organisms, were not recorded. The strain was non-

¹ See Dudgeon: *Lancet*, 1908, 1, p. 615.

pathogenic for rabbits in doses of 1/5 of a slant and did not exhibit specific elective properties for the rabbit urinary system. The patient's serum agglutinated the organism on June 20, 1918, in a dilution of 1:80. This reaction may be interpreted as a slight response to the invasive and pathogenic properties of this organism. Unfortunately no opportunity was afforded to test the patient's serum after vaccination or later in the course of her illness.

As stated in the history, the bacterial flora changed completely after the operation. The anaerogenic, originally also hemophilic, paracolon has disappeared; a typical *B. coli* communior has always and repeatedly been isolated. Many tests have uniformly demonstrated an organisms that behaved biochemically and serologically in an identical manner and characteristic of *B. coli*.

DISCUSSION

We carefully consulted the extensive literature² on the bacteriology of urinary infections. Most of the publications are valueless, the data being collected in a period when blood plates or enrichment in blood broth were not considered necessary as a routine procedure for the study of urinary micro-organisms. Due credit should be given to D. J. Davis,³ who in 1910 called attention to the occasional occurrence of hemophilic bacilli in urine; also to V. C. David⁴ who in a series of 50 urinary cultures derived from diseases of the bladder and kidneys encountered a gram-positive, slightly anaerobic influenza-like bacillus. At first we were inclined to consider the parasitic strain of our bacillus closely related to the one described by Davis. His organism grew only on hemoglobin mediums, the colonies were very minute and opaque and were always hemolytic. The latter features were not observed with our organism; and again the size and tendency for thread formation were more colon-like than diptheroid, as carefully described by Davis for his organism. In many respects our bacillus resembled the *B. pseudo-influenzae* isolated by Wolff⁵ from the bronchi of a rat. Our hemophilic organism, in a manner similar to Wolff's bacillus after repeated transplantations on artificial medium or in the human host subsequent to vaccination, to a clinical relapse or to other unknown factors, reverted "atavistically" to a saprophyte, comparatively easily cultivated on ordinary hemoglobin-free medium. Deprived of its hemophilic tendencies

² Rovsing: Die Infektions-Krankh. der Harnwege, 1899; Koll: Intern. Abstr. of Surgery, 1915, 20, p. 349; Franke: Ergebn. d. Chir. u. Orthopädie 1913, 7, p. 671; Blumenthal and Hammer: Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1908, 18, p. 642; Kodama and Krasnogorski: Centralbl. f. Bakteriöl. I, O., 1913, 69, p. 8; Wulff: Centralbl. f. Bakteriöl. I, O., 1912, 65, p. 27.

³ Jour. Infect. Dis. 1910, 7, p. 599.

⁴ Surg., Gynec. & Obst. 1914, 18, p. 432.

⁵ Centralbl. f. Bakteriöl., I, O., 1903, 33, p. 407.

the bacterium could be readily studied by means of carbohydrate mediums and some suggestions as to its possible classification were obtained. Applying the recent findings of Winslow, Kligler and Rothberg,⁶ Gettings and Inman⁷ and others made with this class of organisms, one would be inclined to place the saprophytic strains with the *B. Flexner-dysenteriae* or *B. gallinarum* group of bacteria. The agglutination and pathogenicity tests and the viscid appearance of the colonies did not, however, justify this conclusion, irrespective of the fact that Foerster⁸ and also Hilgers⁹ quite recently described the findings of true agglutinable dysentery bacilli in the urine. It is not unlikely that the organisms described by the two workers belong to the same group of bacteria as our own. Decidedly more suggestive are the descriptions given by Herrold and Culver¹⁰ for the so-called "paracolon-bacilli." The few carbohydrate reactions recorded by them correspond well with those found for our bacillus. Nongas-producing urinary colon, paracolon or even meta-colon (Jensen-Bahr's classification in Wulff's publication) are not infrequently encountered in urines. Mair,¹¹ Wilson,¹² Sørensen¹³ and Arkwright¹⁴ described such organisms and we therefore decided to place our bacillus tentatively with the paracolon group and designate it as an anaerogenic paracolon bacillus.

The peculiar mucoid, viscid character of the colonies of the saprophytic strains recalled the observations by one of us (K. F. M.)¹⁵ made several years ago on a bacillus (*B. nephritidis-equi* or *B. viscosum-equi* of Magnusson¹⁶) isolated from renal abscesses of a horse. This organism produced such a slimy zooglea-like growth that filaments of from 10 to 20 cm. could be withdrawn on touching with a needle. *B. viscosum* is a colon-like organism; it grows only on glycerol agar medium, produces a toxin and dies out readily on artificial mediums. Degen¹⁷ found a similar organism, which he described under the name of *B. polymorphus-suis*. Unfortunately the descriptions available are

⁶ Jour. Bacteriol., 1919, 4, p. 429.

⁷ Medical Research Committee, Special Report Ser. No. 30, 1919.

⁸ München. med. Wchnschr., 1918, 65, p. 205.

⁹ Centralbl. f. Bakteriöl., I, O, 1919, 83, p. 414.

¹⁰ Jour. Infect. Dis. 1919, 24, p. 114.

¹¹ Brit. Med. Jour. 1906, 1, p. 438.

¹² Jour. Hyg., 1908, 8, p. 543.

¹³ Centralbl. f. Bakteriöl. I, O., 1912, 62, p. 582.

¹⁴ Jour. Hyg., 1913-1914, 13, p. 68.

¹⁵ Report of Government Bacteriologist; Dept. of Agriculture, Pretoria, Transvaal, 1908-1909, p. 122-158.

¹⁶ Jour. Comp. Pathol. & Therap. 1919, 32, p. 143.

¹⁷ Thesis, Giessen, 1907.

incomplete and it would be unwise to consider our bacillus identical with one of these organisms even when some of the characteristics seem to be analogous.

The systematic position of our bacillus is of subordinate interest when we consider more carefully the observation dealing with what was termed parasitic and saprophytic strains. Originally isolated from the urine of a case of hydronephrosis, the bacillus exhibited strict hemophilic properties and distinct capsule formation. These characteristics were subsequently lost on artificial cultivation in vitro and apparently also in the human host. It may be mere coincidence that our bacillus, which originally grew on blood only, developed on plain agar when seeded with urine collected from the patient after she had been vaccinated and had suffered from a relapse. One fact is certain, that systematic urine cultures made this observation possible and materially assisted in the final identification of the organism.

Hemophilic and anaerogenic properties and capsule formation are suggestive, in the sense of Sauerbeck, of a "bacterial immunity by structural adaptation." As already suggested, certain cultural characteristics such as viscosity and loss of gas production are not uncommon with organisms obtained from urinary infections. We have been consulted repeatedly concerning such non-gas producing, slow lactose fermenting colon bacilli isolated from the urine, and we gained the impression that possibly one or several factors as yet incompletely understood or investigated exert a strongly modifying influence on the microbes of the urinary tract. Only in assuming such a condition is it possible to appreciate the fantastic list of bacteria described about 10 years ago by Tanaka.¹⁸ In our particular case the adaptation of our bacillus was not only directed against these rather common influences, but was primarily intended for existences in living tissues. Capsule formation and preference for hemoglobin substrata made their appearance. Removed from the soil to which the organism had been functionally adjusted, and grown on artificial mediums, it gradually reverted atavistically to its ancestral type, namely, a nongas-producing paracolon. It is not unlikely that in the course of time and on suitable mediums, our organism may even acquire the ability to produce gas from some carbohydrates. Such a transformation has been described by Arkwright for a nongas-producing *B. acidilactici* isolated from the urine. And again

¹⁸ Ztschr. f. Urol., 1909, 3, p. 5.

Revis¹⁹ and Penfold²⁰ were able to suppress gas production by the use of chemical—malachite green and chloracetic acid. How far the concomitant *B. alkaligenes* influenced the adaptation of the parasitic strains and how far the vaccination and the frequent clinical recrudescences favored reversion to type cannot be answered definitely. In a paper on irregular typhoid bacilli recently written by one of us (K. F. M.)²¹ attention was called to the importance of functional changes of micro-organisms causing infections in immunized or protected human beings. There are sufficient observations available that clearly indicate that the urinary secretion may exert a “degenerative” or inhibitive influence on the functions of many organisms of the colon group. Studies on bacteria isolated from urinary infections should therefore offer interesting material to the important problem of bacterial variability and adaptation.

SUMMARY

A bacillus isolated from the urine of a case of bilateral infected hydronephrosis is described. It grew as a parasitic capsulated strain only on a medium containing hemoglobin. The bacillus is apparently a member of the paracolon group of bacteria found in urine and is best designated as hemophilic nonaerogenic paracolon. After four months’ cultivation artificially it acquired the property of growing on hemoglobin-free substrata and fermented without gas production the following carbohydrates: hexoses, mannitol, maltose, rhamnose, xylose, arabinose and sorbitol. Samples of urine cultured after the patient had been vaccinated with a heat killed, tricresolized bacterial suspension of the original parasitic strain and after a clinical recrudescence, demonstrated the same organism which grew on nonhemoglobin mediums and which in every respect corresponded with the saprophytic strains obtained by successive cultivation on artificial mediums.

¹⁹ Centralbl. f. Bakteriöl, II, 1911, 31, p. 1.

²⁰ Jour. Hyg., 1912, 12, p. 195.

²¹ Jour. Infect. Dis., 1920, 27, p. 46.

THE DISINFECTION OF THE ORAL MUCOSA WITH CRYSTAL VIOLET AND BRILLIANT GREEN¹

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¹ According to Churchman (*Journal of the American Medical Association*, January 17, 1920), the term "disinfection" when applied to the action of dyes, is a misnomer. He prefers the term "bacteriostasis," not only from an etymological point of view, but also from the standpoint of accuracy of description. We believe, with Churchman, that the term "bacteriostasis" is a more suitable one than "disinfection," in this relation.

I. INTRODUCTION

In the study of the bacteriology of apical and periapical infections, there is great variation in the cultural technique of different investigators. Since the relative value of the results obtained by different experimenters depends upon an unbroken chain of aseptic procedures in each case, it is important to ascertain what technical conditions must be established for the sterilization of the oral mucosa, and also to determine the relative efficiency for this purpose of the available antiseptics. From a consideration of some of the many methods used to obtain material for bacteriological study, especially material in the vicinity of the gingival border, it is apparent that the technique described is not free from criticism. To such an extent does this fact obtain that opinions, rather than scientific facts, appear to have influenced, if not actually to have served as a foundation for, the results reported.

Gilmer and Moody (7) state that "the field to be incised was made as nearly aseptic as possible by thorough washing with 50 per cent alcohol." Schamberg (21) says: "We removed the tooth and from it got a pure culture of *Streptococcus viridans*." Hartzell and Henrici (10) state that it is "almost an impossibility to extract a healthy tooth and find it free from viridans unless that tooth has been rendered free from bacteria by rubbing the tooth with iodine and subsequently burning the gingival field with the actual cautery." Head and Roos (11) "washed the neck of the tooth with alcohol until it was considered to be practically free from living bacteria. It was then painted with tincture of iodine three times at short intervals so that it would be practically impossible for any organisms on the neck of the tooth to be alive." Howe (12) uses two solutions: (a) silver nitrate, 3 gm.; distilled water, 1 cc.; ammonium hydroxid (28 per cent), 2.5 cc. and (b) formalin (25 per cent). These two solutions, mixed, make everything absolutely sterile, according to Howe, and destroy every type of microorganism. Rosenow (20) does not give any technique for obtaining his material but states that the danger of contamination, with the mouth flora, in the removal of teeth, "becomes slight in proper hands."

Kelsey (13) tested a great variety of substances in the culture of organisms to ascertain their value as disinfectants. He concluded that "tincture of iodine is the most useful disinfectant for use in the mouth in treating lesions where strepto-bacillus is present." Kelsey gives no clinical evidence with the results of his laboratory work, but assumes that his laboratory findings apply in vivo as well as in vitro.

II. THE GENERAL PLAN, METHOD, AND RESULTS OF THE AUTHOR'S EXPERIMENTS

In view of the uncertainty revealed by these quoted statements, we sought to find an antiseptic which would be efficient and yet which would be relatively innocuous to the normally sensitive mucous membrane of the oral cavity. In the light of the suggestion by Meyer (19), the problem was attacked from a chemotherapeutic view-point. Accordingly, observations were made upon the therapeutic and chemical effects produced by various drugs ordinarily used in clinical practice, as well as upon suggested changes in concentration of the antiseptics. The mode of application was the same in all instances, namely, by means of cotton pellets moistened with the different fluids. Application was always made on the gum of the maxilla near the frenum.

1. First series. Results with alcohol, acetone, ether, and tincture of iodine

The results obtained with alcohol, acetone, ether, tincture of iodine, and *dilute* tincture of iodine, are summarized in *table 1*. It will be noted that, in the use of either 60 per cent alcohol or 95 per cent alcohol, there was variation in the degree of dehydration of the gum. When the application of alcohol was followed, in turn, first by ether, second by acetone, finally by a mixture of the three in equal proportions, the results varied in the degree of dehydration induced, as well as in the degree of sterility of the tissue. In each case there was a definite dehydrating action on the gum by the alcohol, an effect that renders the use of alcohol of these strengths unsuitable for all practical purposes of oral disinfection, even though in some tests

sterility was obtained in 100 per cent of the tests. Tincture of iodine, also dilute tincture of iodine (1:10), was used. With the former *sterility was obtained, but only at the expense of blistering the gum surface. With the latter practically no sterility was obtained.*

TABLE 1

Data on the efficiency of alcohol, ether, acetone, and iodine in efforts to obtain sterility in the buccal cavity.

DRUGS TESTED	PERIOD OF APPLICATION	NUMBER OF CASES	BACTERIOLOGICAL RESULT	EFFECTS ON THE GUMS
	<i>minutes</i>			
Alcohol, 95 per cent	2	5	Sterility in but one case	Gums badly dehydrated
Alcohol, 95 per cent	3	5	Sterility in four cases	Gums badly dehydrated
Alcohol, 60 per cent	2	7	No sterility	Gums not dehydrated
Alcohol, 60 per cent	3	5	No sterility	Gums slightly dehydrated
Alcohol (95 per cent), ether, and acetone, in one-two-three-order	1, for each substance	5	No sterility	Gums badly dehydrated
Alcohol (95 per cent), ether, and acetone, in one-two-three order	2, for each substance	5	Sterility in but two cases	Gums badly dehydrated
Alcohol (95 per cent), ether, and acetone, mixed in equal proportions	2	4	Sterility in but two cases	Gums badly dehydrated
Tincture of iodine	1	3	Sterility in three cases	Gums dehydrated and "burned"
Tincture of iodine, diluted with water (1:10)	1	3	No sterility	Gums apparently not "burned"
Tincture of iodine, diluted with water (1:10)	2	3	Sterility in one case	Gums "burned"
Total		45	Sterility in thirteen cases	

Seelig and Gould (22) as early as 1911, definitely proved that alcohol is a good antiseptic in direct proportion to its concentration. The results of our experiments show that the utility of alcohol as an antiseptic, in the strengths referred to (60 per cent and 95 per cent),

is impaired, for oral purposes, by its undesirable dehydrating action on the mucous membranes—one of the effects we have endeavored to overcome. Such dehydration of the oral mucous membranes is objectionable because blistering or burning of the gum follows it, and the ensuing lowered local resistance and subsequent superficial necrosis delay the process of healing and favor the further ingress of microorganisms.

2. Second series. Results with dyes

A. Historical

Leitch (14) used brilliant green in dilute aqueous solution. He found it non-toxic to normal tissue, though antiseptic in action. He also observed that this dye inhibited the growth of staphylococci to a greater extent than it did that of streptococci. The same dye was used, as a paste for injection into wounds, by Short (23) who obtained equally good results. Browning (5) found that brilliant green, 1:2000 in water, is five hundred times as toxic for staphylococci as is Dakin's solution. He also found (4) that it stimulates healthy granulation tissue as well as acts as an antiseptic. Webb (24) and Ligot (15) obtained similar results with the dye.

Browning (2, 1, 3) found that a mixture of crystal violet and brilliant green kills gram-positive cocci chiefly, whereas *B. coli*, resists its action to a greater extent. He used a solution of 1 per cent of crystal violet and 1 per cent of brilliant green, together in 50 per cent alcohol, with excellent results, and suggested the use of the dye as an antiseptic for mucous membranes.

Graham-Smith (8) found that the toxicity or inhibitory power of crystal violet, as well as various flavines [acriflavine and proflavine, which are acridine dyes, derivatives of acridine, a base found in coal tar (9)], depends to a great extent upon the degree of alkalinity of the solution to which the dye is added—that is, the greater the alkalinity, to a certain degree, the greater is the antiseptic power. For example, crystal violet inhibits the growth of *Staphylococcus aureus* under the conditions indicated on the next page:²

²The ratio signifies, in each case, that one part of the dye in the indicated number of parts of aqueous solution, with the reaction stated, is the greatest dilution of the dye, in the particular circumstances, that will completely prevent growth of the organism named.

<i>Dye tested and medium used</i>	<i>0.15 n/10 hydrochloric acid</i>	<i>0.08 n/10 sodium hydroxid</i>	<i>0.58 n/10 sodium hydroxid</i>
	1:1,500,000	1:3,500,000	1:5,000,000
	<i>Organisms studied</i>		
	<i>Staphylococcus</i>	<i>B. coli</i>	<i>B. pyocyaneus</i>
Crystal violet			
Agar.....	1:3,500,000	1:10,000	1:10,000
Broth.....	1:10,000,000		
Brilliant red			
Agar.....	1:3,250,000	1:1,000	1:1,000
Broth.....	1:500,000	1:10,000	1:10,000
Quinone			
Agar.....	1:10,000	1:95,000	1:30,000
Broth.....	1:60,000	1:100,000	1:10,000
Hydroquinone			
Agar.....	1:60,000	1:40,000	1:10,000
Broth.....	1:20,000	1:20,000	1:10,000
Homoflavine			
Agar.....	1:14,000	1:9,000	1:2,000
Broth.....	1:300,000	1:250,000	1:30,000

Thus we note that in alkaline media, the dye tends to be more efficient as an antiseptic, at least so far as *Staphylococcus aureus* is concerned.

According to Marshall (18) the hydrogen ion concentration of saliva varies not only in different individuals, but also in the same individual from time to time. This variation, as has been pointed out by other investigators, may be due to nervous impulses or reflexes which appear to be correlated with such factors as diet, and with the pathology of the oral and gastric mucosae and the teeth.

A small series of experiments was undertaken by us to determine the hydrogen ion concentration (P_H) of saliva by the colorimetric method (with phenolsulfonephthalein as the indicator), according to the procedures of Clark and Lubs (6) and of MacLeod (17), with the following results for P_H in six different specimens: 7.3, 7.3, 7.8, 7.6, 7.3, 7.5; average, 7.43.

The average for P_H , 7.43, indicates a fair degree of alkalinity, which appears to be sufficient to enhance the value of the dye (crystal violet) as an antiseptic.

B. Technical

The solutions to be tested were applied to the gum of the upper jaw between the mucosa of the lip and of the gum beside the frenum, because of the ease with which one may eliminate saliva from the field

by packing with sterile cotton. The dye was applied by means of pledgets of cotton saturated with the dye solution of various strengths and for different periods. Bacterial cultures were made in glucose-veal bouillon, and on glucose-blood agar slants or plates, by the use of sterile swabs, kept in the individual test tubes in which they were sterilized.

C. Results with brilliant green (saturated solution in alcohol)

A saturated solution of brilliant green³ in 95 per cent alcohol or in 60 per cent alcohol, was applied, as indicated above, and the swab cultured after one minute of application. A second test was subse-

TABLE 2

Data on the efficiency of alcoholic solutions of brilliant green in efforts to obtain sterility in the buccal cavity

SOLUTION OF BRILLIANT GREEN	PERIOD OF APPLICATION	NUMBER OF CASES	BACTERIOLOGICAL RESULT	EFFECTS ON THE GUMS
	<i>minute</i>			
Saturated, in 95 per cent alcohol	1	5	Sterility in five cases	Gums badly dehydrated
Saturated, in 60 per cent alcohol	1	5	Sterility in five cases	Gums only slightly dehydrated
Saturated, in 95 per cent alcohol	1	5	Sterility in five cases	Gums very badly dehydrated
Followed by washing with 95 per cent alcohol	0.5			
Saturated, in 60 per cent alcohol	1	5	Sterility in five cases	Gums only slightly dehydrated
Followed by washing with 60 per cent alcohol	0.5			
Total.....		20	Sterility in twenty cases	

quently made in which the excess of the dye remaining on the gum was removed by washing with alcohol of the same strength as that used originally to dissolve the dye. The part so treated was then tested for sterility in the usual manner. Sterility was obtained in all cases,

³ Thus far only Gr bler's dyes have been used in this work.

but the alcohol exerted too great a dehydrating action upon the mucous membrane to permit of its routine application. The results are summarized in *table 2*.

D. Results with brilliant green (1 per cent solution in 50 per cent alcohol)

In this group of tests the technique was similar to that of the tests in section C, except that we used a 1 per cent solution of brilliant green in 50 per cent alcohol. Sterility was obtained in 78.5 per cent of the cases and there was only a slight degree of harmful effect of the alcohol upon the gums. The results are shown in *table 3*.

TABLE 3

Additional data on the efficiency of brilliant green in alcoholic solution in efforts to obtain sterility in the buccal cavity

SOLUTION OF BRILLIANT GREEN	PERIOD OF APPLICATION	NUMBER OF CASES	BACTERIOLOGICAL RESULT	EFFECTS ON THE GUMS
	<i>minute</i>			
1 per cent in 50 per cent alcohol	1	7	Sterility in five cases	Gums very slightly dehydrated
1 per cent in 50 per cent alcohol	1	7	Sterility in six cases	Gums very slightly dehydrated
Followed by washing with 50 per cent alcohol	0.5			
Total.....		14	Sterility in eleven cases (78.5 per cent)	

E. Results with brilliant green and crystal violet in solution *together* (1 per cent of each in 50 per cent alcohol) applied for 1.5 minute

In this series of cases, the dye employed was a mixture of brilliant green and crystal violet, 1 per cent of each in 50 per cent alcohol, applied for 1.5 minute. The action of the dye upon the organisms normally present was measured, in this instance, by taking cultures (a) from the gum-surface before applying the dye, and (b) from the relative numbers of organisms computed to be present in smears of the broth culture, as well as (c) from the number of colonies on plates of enrichment media.

TABLE 4

Data on the efficiency of crystal violet and brilliant green together (1 per cent of each), in 50 per cent alcohol, in efforts to obtain sterility in the buccal cavity. Period of application to the gum: 1.5 minute. (In this, and succeeding tables, the signs, x, xx, and xxx, indicate the relative numbers of organisms in the cultures. Where no such sign is recorded, a pure culture was involved)

CASE NUMBER	ORGANISMS ON THE GUM-SURFACE BEFORE THE DYE WAS APPLIED	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED	RESULTS AFTER THE EXCESS OF THE DYE HAD BEEN REMOVED BY WASHING WITH ALCOHOL	TYPE OF CASE
17	{ Streptococci xxx Diplostreptococci x	Sterile in 48 hours	Same	Pyorrhea
18	{ Diplostreptococci xxx Streptococci, short chain x Staphylococcus albus x	Sterile in 48 hours	Same	Pyorrhea
19	{ B. acidophilus xxx Diplostreptococci xx Streptococci x	Diplostreptococci present	Same	Pyorrhea
20	{ Diplostreptococci xxx Streptococci xx Staphylococcus albus x	Sterile in 36 hours	Same	Pyorrhea
21	{ Diplostreptococci xxx Staphylococcus albus x Streptococci x	Sterile in 36 hours	Same	Pyorrhea
22	{ Diplostreptococci xxx Streptococci x	Sterile in 36 hours	Same	Pyorrhea
23	{ Diplostreptococci xxx Staphylococcus albus x	Sterile in 36 hours	Same	Pyorrhea
24	{ Diplostreptococci xxx Streptococci x	Sterile in 36 hours	Same	Pyorrhea
25	{ Streptococci, long chain xxx Staphylococcus albus xxx	Streptococci, long chain present	Same	Pyorrhea
26	{ Streptococci xxx Diplostreptococci x	Sterile	Same	Pyorrhea
27	{ Staphylococcus albus xxx Streptococci, short chain xxx	Sterile in 48 hours	Same	Pyorrhea

TABLE 4—*Concluded*

CASE NUMBER	ORGANISMS ON THE GUM-SURFACE BEFORE THE DYE WAS APPLIED	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED	RESULTS AFTER THE EXCESS OF THE DYE HAD BEEN REMOVED BY WASHING WITH ALCOHOL	TYPE OF CASE
28	{ B. acidophilus xx Streptococci, short chain xx Staphylococcus albus x	Gram-rod-like organism present	Same	Pyorrhea
29	{ Diplostreptococci xxx Streptococci, short chain xx	Sterile in 48 hours	Same	Pyorrhea
30	{ Streptococci, long chain xx Staphylococcus aureus x	Sterile in 36 hours	Same	Pyorrhea
Total number of cases, 14. Sterility in the number of cases indicated below:				
		11 cases; 78.5 per cent	10 cases; 75 per cent	

By comparing these results with those obtained *after* swabbing the gum-surface with the dye, and after the subsequent removal of the excess of the dye with 50 per cent alcohol, a direct determination of the sterilizing action of the fluids could be made. The cases were all diagnosed as pyorrheal. Sterility was obtained in 75 per cent of them. The results are given in *table 4*.

F. Results of tests similar to those in the last preceding section (E), with additional bacteriological precautions

In addition to the previous precautions and technique, this series of experiments was further controlled by planting the last swab, used in culturing the gum-surface, in glucose-veal broth. In this series of tests we obtained sterility in 80 per cent of the cases. The findings are given in *table 5*.

TABLE 5

Additional data on the efficiency of crystal violet and brilliant green together (1 per cent of each), in 50 per cent alcohol, in efforts to obtain sterility in the buccal cavity. Period of application to the gum: 1.5 minute.

CASE NUMBER	ORGANISMS ON THE GUM-SURFACE BEFORE THE DYE WAS APPLIED	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED	RESULTS AFTER THE EXCESS OF THE DYE HAD BEEN REMOVED BY WASHING WITH ALCOHOL	RESULT IN THE CULTURE OF THE LAST SWAB	TYPE OF CASE
31	Streptococci xxx Diplostreptococci x Staphylococci x	Present: Streptococci xx Diplostreptococci x	Sterile	Sterile	Pyorrhea
32	Streptococci xx Staphylococci x	Sterile in 48 hours	Staphylococcus albus present	Staphylococci present	Pyorrhea
33	Diplostreptococci xxx	Sterile in 48 hours	Same	Same	Pyorrhea
40	Streptococci, long chain xx	Sterile in 36 hours	Same	Same	Pyorrhea
41	Streptococci, long chain xxx Diplostreptococci x	Sterile in 36 hours	Same	Same	Pyorrhea
42	Gram-rod (B. coli) xxx Staphylococci xx	B. coli present	B. coli present	B. coli present	Pyorrhea
43	Streptococci, short chain xxx Staphylococci x Streptococci, long chain x Diplostreptococci x	Sterile in 48 hours	Same	Same	Pyorrhea
46	Staphylococcus albus xx Streptococci x	Sterile in 48 hours	Same	Same	Pyorrhea
47	Diplostreptococci xx Streptococci, short chain x	Sterile in 48 hours	Same	Same	Pyorrhea
49	Diplostreptococci xxx Streptococci x Staphylococcus albus x	Sterile in 48 hours	Streptococci present	Sterile in 48 hours	Pyorrhea
Total number of cases, 10. Sterility in the number of cases indicated below:					
		8 cases; 80 per cent	7 cases; 70 per cent	8 cases; 80 per cent	

TABLE 6

Data on the comparative efficiency of crystal violet and brilliant green together (1 per cent of each) and of hydroquinone (1 per cent) in aqueous solution, in efforts to obtain sterility in the buccal cavity. Periods of application to the gum: (a) crystal violet and brilliant green, 2 minutes, or 2.5 minutes; (b) hydroquinone, 2.25 minutes. Type of cases: all pyorrhea

CASE NUMBER	ORGANISMS ON THE GUM-SURFACE BEFORE THE DYE WAS APPLIED	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED FOR 2 MINUTES	RESULT IN THE LAST CORRESPONDING SWAB CULTURE	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED FOR 2.5 MINUTES	RESULT IN THE LAST CORRESPONDING SWAB CULTURE	RESULTS AFTER THE HYDRO-QUINONE SOLUTION HAD BEEN APPLIED FOR 2.25 MINUTES
60	Streptococcus xxx Diplostreptococcus x	Same	Same	Same	Same	Same
61	Streptococcus xx Diplostreptococcus x Staphylococcus albus x	Streptococcus present	Present: Streptococcus xx Diplostreptococcus x	Streptococcus present	Streptococcus present	Same as control (first column)
62	Streptococcus x Diplostreptococcus x	Same	Same	Same	Same	Same
63	Staphylococcus xxx Streptococcus xx	Streptococcus present	Present: Streptococcus xx Staphylococcus x	Streptococcus present	Streptococcus present	Same as control (first column)
64	Streptococcus xx B. coli x	Same	Same	Same	Same	Same
65	Streptococcus	Same	Same	Same	Same	Same
66	Streptococcus xx Staphylococcus xx Diplostreptococcus x	Present: Streptococcus x Staphylococcus x	Present: Streptococcus x Staphylococcus x Diplostreptococcus x	Present: Streptococcus x Staphylococcus x	Same	Same as control (first column)
67	Staphylococcus xx Streptococcus x	Same	Same	Same	Same	Same

TABLE 6—*Concluded*

CASE NUMBER	ORGANISMS ON THE GUM-SURFACE BEFORE THE DYE WAS APPLIED	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED FOR 2 MINUTES	RESULT IN THE LAST CORRESPONDING SWAB CULTURE	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED FOR 2.5 MINUTES	RESULT IN THE LAST CORRESPONDING SWAB CULTURE	RESULTS AFTER THE HYDROQUINONE SOLUTION HAD BEEN APPLIED FOR 2.25 MINUTES
68	Diplostreptococcus xx Streptococcus x	Same	Same	Same	Same	Same
69	Streptococcus xx B. coli xx Diplostreptococcus x Staphylococcus x	Same	Same	Present: Streptococcus xx B. coli xx Diplostreptococcus x	Same	Same as control (first column)

G. Results of tests with brilliant green and crystal violet in solution *together* (1 per cent of each in aqueous solution) compared with hydroquinone (1 per cent aqueous solution)

At this stage we considered it advisable to ascertain the relative value of an *aqueous* solution of the dyes compared with an aqueous solution of hydroquinone. Accordingly we made a solution of brilliant green and crystal violet (1 per cent of each, together), and another of hydroquinone (1 per cent), in sterile distilled water. The bacteriological technique was the same as that outlined for the tests in section F.

The results, as shown in *table 6*, were wholly unsatisfactory, from an experimental point of view, for practically no sterility was obtained under the conditions of the tests.

H. Results of a repetition of the tests with brilliant green and crystal violet in solution *together* in alcohol (section E), with an extension of the period of application to the gum (from 1.5 minute) to 2 minutes

We repeated the technique of the tests in section E (with 1 per cent each of brilliant green and crystal violet together in solution in 50 per cent alcohol), but allowed a little more time for the action of the

TABLE 7

Additional data on the efficiency of crystal violet and brilliant green together (1 per cent of each), in 50 per cent alcohol, in efforts to obtain sterility in the buccal cavity. Period of application to the gum: 2 minutes

CASE NUMBER	ORGANISMS ON THE GUM-SURFACE BEFORE THE DYE WAS APPLIED	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED	RESULT IN THE 1st CULTURE OF THE LAST SWAB	TYPES OF CASES
65/2	{ Streptococcus xx Diplostreptococcus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
66/2	Streptococcus	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
67/2	{ Diplostreptococcus xx Streptococcus	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea and caries
68/2	{ Staphylococcus albus xx Streptococcus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
69/2	Streptococcus	Sterile in 48 hours	Sterile in 48 hours	Edentulous
70	{ Diplostreptococcus x Staphylococcus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
71	{ Diplostreptococcus xx Streptococcus x Staphylococcus albus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
72	{ Streptococcus, long chain xx Streptococcus, short chain xx Staphylococcus x Diplostreptococcus x	Streptococcus, long chain, present	Streptococcus, long chain, present	Pyorrhea
73	Staphylococcus albus	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
75	{ Streptococcus x Diplostreptococcus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
76	{ Diplostreptococcus xx Staphylococcus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
77	{ Diplostreptococcus xxx Staphylococcus x Streptococcus, long chain x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea

TABLE 7—Continued

CASE NUMBER	ORGANISMS ON THE GUM-SURFACE BEFORE THE DYE WAS APPLIED	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED	RESULT IN THE CULTURE OF THE LAST SWAB	TYPE OF CASES
78	{ Streptococcus xxxx Diplostreptococcus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea and caries
79	{ Streptococcus xx Staphylococcus albus x Diplostreptococcus x	Staphylococcus albus present	Staphylococcus albus present	Pyorrhea
80	Streptococcus, long chain	Sterile	Sterile	Edentulous
81	{ Diplostreptococcus xx Streptococcus x	Sterile	Sterile	Advanced caries
85	{ Staphylococcus albus x Diplostreptococcus x	Sterile	Sterile	Pyorrhea
86	{ Streptococcus xxx Diplostreptococcus x	Sterile	Sterile	Pyorrhea
87	{ Diplostreptococcus xx Streptococcus x Staphylococcus albus x	Sterile	Sterile	Pyorrhea
88	{ Staphylococcus albus xx Streptococcus x	Sterile	Staphylococcus albus present	Pyorrhea
89	Streptococcus	Sterile	Sterile	Pyorrhea
90	{ Diplostreptococcus xx Streptococcus x	Sterile	Sterile	Pyorrhea
92	{ Streptococcus, long chain xx Streptococcus, short chain x Staphylococcus albus x	Sterile	Sterile	Fairly clean mouth
93	{ Diplostreptococcus xxx Streptococcus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
94	Diplostreptococcus	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea

TABLE 7—*Concluded*

CASE NUMBER	ORGANISMS ON THE GUM-SURFACE BEFORE THE DYE WAS APPLIED	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED	RESULTS IN THE CULTURE OF THE LAST SWAB	TYPES OF CASES
95	{ Streptococcus xxx Staphylococcus xx Diplostreptococcus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
96	{ Streptococcus x Diplostreptococcus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
97	{ Staphylococcus albus xx Streptococcus x	Sterile in 48 hours	Sterile in 48 hours	Pyorrhea
98	{ Streptococcus xxx Diplostreptococcus xx	Streptococcus present	Streptococcus present	Pyorrhea
99	{ Diplostreptococcus xx Streptococcus x	Streptococcus present	Streptococcus present	Pyorrhea
101	{ Streptococcus x Staphylococcus albus x	Streptococcus present	Streptococcus present	Pyorrhea
102	{ Streptococcus	Streptococcus present	Streptococcus present	Pyorrhea
103	{ Streptococcus xx Diplostreptococcus x	Streptococcus present	Streptococcus present	Pyorrhea
104	{ Diplostreptococcus xxx Staphylococcus albus x Streptococcus x B. coli xx	B. coli present	B. coli present	Pyorrhea
105	{ Streptococcus xx Staphylococcus albus x	Sterile	Sterile	Pyorrhea
106	{ Streptococcus xxx Staphylococcus albus xx	Sterile	Sterile	Pyorrhea
108	{ Diplostreptococcus xxx Streptococcus x	Sterile	Sterile	Pyorrhea

dye, namely, 2 minutes instead of 1.5 minute. The results in *table 7* are summarized below:

	Number	Per cent
Total number of cases cultured.....	37	
Sterile after application of the dye for two minutes.....	33	89.2
Sterile swabs.....	32	86.9
Staphylococcus persisted after the application of the dye.....	1	2.8
Streptococcus persisted after the application of the dye.....	1	2.8

In this series of tests, and in those of the preceding groups, we noted that *B. coli* was not affected by the dye to any appreciable extent. We endeavored, then, to ascertain the comparative effects of the dye on *B. coli* and on a streptococcus. See the experiment described in the footnote.⁴

⁴ The supernatant fluid from a 100 cc. glucose-blood veal-broth culture of a streptococcus isolated from an apical infection was carefully pipetted off and centrifuged. The organisms thus obtained were suspended in normal salt solution and half their number, 500 billions, injected into the ear veins of a rabbit. The rabbit was sacrificed in ten minutes, immediately placed in 1:1000 lysol solution, and carefully autopsied. Portions of the liver, lungs, heart, and kidneys were obtained aseptically and placed in sterile petri dishes. Several pieces of each of these tissues, 0.5 x 0.5 x 1 cm. in size, were dipped into a suspension of *B. coli*. One of these pieces was then immersed for five seconds in paraffin oil at 180°C., when it was macerated in a sterile mortar in broth and sand, and plated in glucose-blood agar; also in Endo's medium according to the method of McMaster (16). Others of the pieces treated with *B. coli* were dipped in a solution of brilliant green and crystal violet together (1 per cent each) in 50 per cent alcohol for 1.5 minute and the excess of the dye washed off with 50 per cent alcohol for 0.5 minute. The tissue was finally macerated as in the case of the pieces immersed in paraffin oil, with the following results:

TISSUE TREATED WITH <i>B. COLI</i>	AFTER IMMERSION IN PARAFFIN OIL AT 180°C. FOR 5 SECONDS		AFTER APPLICATION OF THE DYE SOLUTION — CRYSTAL VIOLET AND BRILLIANT GREEN TOGETHER (1 PER CENT OF EACH) IN 50 PER CENT ALCOHOL — FOR 1.5 MINUTE FOLLOWED BY WASHING WITH 50 PER CENT ALCOHOL FOR 0.5 MINUTE	
	Organisms in Endo's medium	Organisms in the glucose-blood broth	Organisms in Endo's medium	Organisms in the glucose-blood broth
Heart.....	No growth	Streptococci	<i>B. coli</i>	Streptococci and <i>B. coli</i>
Kidney.....	No growth	Streptococci	<i>B. coli</i>	Streptococci and <i>B. coli</i>
Lungs.....	No growth	Streptococci	No growth	Streptococci
Liver.....	No growth	Streptococci	No growth	Streptococci

These tests show that in the second case, i.e., with the dye, there was no growth of *B. coli* in the cultures from the lungs and liver, possibly because of the better penetration of the dye into the lungs and liver, as a consequence of the peculiarities of the anatomical structure of these tissues.

I. Results of a repetition of the tests with brilliant green and crystal violet *together* in alcohol, in the last preceding section (H), after previous thorough flushing of the mouth with an alkaline wash and brushing of the teeth

After carefully flushing the mouth with an alkaline wash and brushing the teeth, the technique for the tests in section H was used, with the results shown in *table 8*.

TABLE 8

Additional data on the efficiency of crystal violet and brilliant green together (1 per cent of each), in 50 per cent alcohol, in efforts to obtain sterility in the buccal cavity. Period of application to the gum: 2 minutes (after flushing the mouth with an alkaline wash and brushing the teeth)

CASE NUMBER	ORGANISMS ON THE GUM-SURFACE BEFORE THE DYE WAS APPLIED	RESULTS AFTER THE DYE SOLUTION HAD BEEN APPLIED	RESULT IN THE CULTURE OF THE LAST SWAB	TYPES OF CASES
110	Streptococcus	Sterile	Sterile	Pyorrhea
111	Streptococcus	Sterile	Sterile	Pyorrhea
112	Staphylococcus	Sterile	Sterile	Pyorrhea
113	{ Streptococcus x Diplostreptococcus	Sterile	Sterile	Pyorrhea
114	Streptococcus	Sterile	Sterile	Pyorrhea
115	Diplostreptococcus	Sterile	Sterile	Pyorrhea
116	{ Diplostreptococcus xx Streptococcus x	Sterile	Sterile	Extensive caries
117	Diplostreptococcus	Sterile	Sterile	Pyorrhea
118	Streptococcus	Streptococcus present	Sterile	Pyorrhea
119	{ Diplostreptococcus xx Streptococcus x	Sterile	Sterile	Pyorrhea
120	Staphylococcus	Sterile	Sterile	Pyorrhea
121	{ Streptococcus xxx Diplostreptococcus x	Sterile	Sterile	Pyorrhea
122	Streptococcus	Sterile	Sterile	Pyorrhea
123	{ Streptococcus, long chain x Streptococcus, short chain x	Sterile	Sterile	Pyorrhea

Total number of cases: 14; sterility in 13 cases, or 92 per cent; absolute sterility in 14 cases or 100 per cent.

3. General discussion of the results of the first two series of tests

Alcohol and iodine should be rejected as oral antiseptics on account of their harmful effects on the delicate mucous membrane despite their apparently good qualities as antiseptics elsewhere.

Since neither crystal violet nor brilliant green is much affected by the oral secretions, and as the action of each is enhanced by the slight alkalinity of the saliva, we regard these dyes as excellent oral antiseptics, in accord with the findings recorded in sections D, E, F, H, and I above.

The results of the tests described in section I, show that advantage may be derived from a thorough cleansing of the mouth and teeth before applying the antiseptic. The alkaline mouth-wash employed in the tests for this purpose appears to have been responsible for the improvement of the results (I).

4. *Third series. Clinical findings*

The results of the foregoing experiments show the efficiency of dyes as antiseptics from the laboratory view-point. Consequently, we endeavored to ascertain their value from the clinical and therapeutic aspects.

The following case histories have been selected from a very large number to *illustrate* the effects of practical application, in dental surgery, of crystal violet and brilliant green in solution together (1 per cent of each in 50 per cent alcohol), referred to below as "the dye."

Case 1. Mr. J. A. An extensive bone cyst in the mandible. It was opened, curetted, drained, and treated with the dye. The discharge, which had been profuse and purulent, became scanty and serous after two applications with a one-day interval. Granulations began to grow inward from all sides. The wound gradually closed up in four weeks of this treatment.

Case 2. Mr. M. S. A rather large granuloma, and surrounding infected bone, was incised, curetted, drained, and treated with the dye. Granulations grew rapidly and healing was by first intention.

Case 3. Mr. J. F. An osteomyelitis of the lower third of a fibula. It was opened, drained, and treated with camphor-phenol for one month without improvement and with a continuing profuse, purulent, foul discharge. The dye was applied and the discharge soon became scanty, serous and odorless. Two small sequestra were recently removed. Healing began promptly after the operation and is proceeding rapidly.

Case 4. Mrs. J. A surgical removal of granulomata and the surrounding necrotic bone. The wound was treated with the dye. Healing took place promptly, with no discharge save in one area, which is yielding rapidly to treatment with the dye.

Case 5. Mr. J. H. A severe case of pyorrhea. Teeth were removed. The wounds were treated with the dye three times. There has been no history of pain or soreness. Healing progressed at a rapid rate.

Case 6. Mrs. B. Extensive apical and periapical infections. Teeth were surgically removed. The wounds were treated once weekly, for three weeks, with the dye. Healing was complete with almost no discharge. Dentures were constructed in the third week after the extractions.

Case 7. Mr. R. A superior first molar was removed surgically under local anesthesia. A severe case of eburnated bone. Treated twice with the dye. Healing was complete in five days, with no discharge.

Case 8. Miss C. Granulomata and necrosis about lower first and second molars. These were removed under local anesthesia, with extensive curettage. The wounds were treated daily, for a week, with the dye. There were slight, scanty, serous discharges, but no pain. The wounds were completely healed in three weeks.

Many other cases might be mentioned, in which the results were quite similar. In the research laboratories and clinics of the dental department a large number of patients have been treated with the dye. The histories of these cases appear to confirm the general deduction stated above regarding the antiseptic value of the dye. The improvement in cases of chronic gingivitis has been very marked. Injection of the dye into pyorrheal pockets is of only temporary benefit, however, for the tendency toward tissue degeneration is not noticeably altered.

Discussion of the clinical findings

Solutions of crystal violet and brilliant green may be easily applied to the gums and even deeper structures in the oral cavity. The diffusion and penetration of the dye is considerable. The clinical data reported above are wholly in keeping with the results that various observers have obtained with dyes as antiseptics for other parts of the body. In every instance, in our experience, the case was benefited by treatment with the dye. Extractions, old discharging sinuses and old infections, yield completely to treatment.

Solutions of crystal violet and brilliant green appear to have no lasting beneficial effect upon pyorrhea. The only result we obtained was merely a temporary diminution in the severity of the symptoms.

III. SUMMARY OF GENERAL CONCLUSIONS

Brilliant green and crystal violet, in solution together (1 per cent of each in 50 per cent alcohol), are very efficient, mild antiseptics. This combination has very little, if any, harmful effect on the delicate mucous membrane of the mouth.

Clinical observations indicate that these dyes together in alcohol act not only as an antiseptic, but also as a mild stimulant to newly forming granulations, both by keeping down contamination by oral organisms and by either destroying or inhibiting the growth of organisms initially present.

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LITERATURE CITED

- (1) BROWNING, C. H. 1918 Applied bacteriology Oxford Press, London, England.
- (2) ———, AND BONNEY, V. 1918 Sterilization of the skin and other surfaces by a mixture of crystal violet and brilliant green. *British Medical Journal*, p. 562 (part 1).
- (3) ———, AND GULBRANSEN, G. 1919 The testing of antiseptics in relation to their use in wound treatment. *Journal of Hygiene*, xviii, p. 33.
- (4) ———, GULBRANSEN, R., KENNAWAY, E. L., AND THORNTON, L. H. D. 1917 Flavine and brilliant green. *British Medical Journal*, p. 73 (part 1).
- (5) ———, GULBRANSEN, R., and THORNTON, L. H. D. 1917 The antiseptic properties of acriflavine and proflavine and brilliant green. *British Medical Journal*, p. 70 (part 2).
- (6) CLARE, W. M., AND LUBS, H. A. 1917 The colorimetric determination of hydrogen ion concentration and its application to bacteriology. *Journal of Bacteriology*, ii, p. 191.
- (7) GILMER, T. L., AND MOODY, A. M. 1914 A study of the bacteriology of alveolar abscess and infected root canals. *Journal of the American Medical Association*, lxiii, p. 2023.
- (8) GRAHAM-SMITH, G. S. 1919 Some factors influencing the action of dyes and allied compounds on bacteria. *Journal of Hygiene*, xviii, p. 1.
- (9) ———. 1919 New and non-official remedies: acriflavine and proflavine. *Journal of the American Medical Association*, lxxiii, p. 1443.

- (10) HARTZELL, T. B., AND HENRICI, A. T. 1915 Oral prophylaxis in its relation to pyorrhea and its treatment. *Journal of the National Dental Association*, ii, p. 122.
- (11) HEAD, J., AND ROOS, C. 1919 On the bacteriology of apical abscesses. *Journal of Dental Research*, i, p. 13.
- (12) HOWE, P. R. 1919 Dental foci. *Dental Summary*, xxxix, p. 793.
- (13) KELSEY, C. J. 1919 Oral bacteria exhibiting strepto-bacillary characters. *British Dental Journal*, xl, p. 373.
- (14) LEITCH, A. 1916 Brilliant green as an antiseptic. *British Medical Journal*, p. 236 (part 1).
- (15) LIGOT, D. 1917 Flavine and brilliant green in the treatment of infected wounds. *British Medical Journal*, p. 73 (part 1).
- (16) MCMASTER, P. D. 1919 The germicidal power of antiseptic oils and of substances dissolved in oils. *Journal of Infectious Diseases*, xxiv, p. 378.
- (17) MACLEOD, J. J. R. 1919 The diagnosis of acidosis. *Journal of Laboratory and Clinical Medicine*, iv, p. 315.
- (18) MARSHALL, JOHN A. 1915 The neutralizing power of saliva in its relation to dental caries. *American Journal of Physiology*, xxxvi, p. 260.
- (19) MEYER, K. F. 1917 The present status of dental bacteriology. *Journal of the National Dental Association*, iv, p. 966.
- (20) ROSENOW, E. C. 1919 Studies on elective localization. Focal infection, with special reference to oral sepsis. *Journal of Dental Research*, i, p. 205; also *Journal of the National Dental Association*, vi, p. 983.
- (21) SCHAMBERG, M. I. 1914 Abstract of a discussion of a symposium on mouth infections. *Journal of the American Medical Association*, lxiii, p. 2029.
- (22) SEELIG, M. G., AND GOULD, C. W. 1911 Osmosis as an important factor in the action of antiseptics. *Surgery, Gynecology and Obstetrics*, xii, p. 262.
- (23) SHORT, A. R., ARKLE, J. S., AND KING, C. 1917 Report on wound treatment by brilliant green paste. *British Medical Journal*, p. 506 (part 2).
- (24) WEBB, C. H. S. 1917 A note on the value of brilliant green as an antiseptic. *British Medical Journal*, p. 870 (part 1).

XVIII. CONDUCTION IN THE SMALL INTESTINE

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During the last year we have brought forward considerable evidence in favor of the view that there is a metabolic gradient in the intestinal wall from duodenum to ileum (1). Per unit of weight and time the duodenal muscle gives off more CO_2 than does the ileal muscle. That this is not due simply to its greater activity is shown by the fact that the difference can still be shown when both segments are kept paralyzed by adrenalin. It is our belief that this metabolic gradient underlies and gives rise to gradients of rhythmicity, latent period and irritability which determine the direction of the diastaltic waves.

It next occurred to us that such a metabolic gradient might influence the conduction of stimuli up and down the intestine. We should expect such stimuli to travel farther and faster with the gradient than against it. It seemed worth while to look into the matter not only because of its academic interest but because the demonstration of such differences in conduction might throw light upon the mechanism of the "myenteric reflex" and upon various clinical problems. When we contemplate the tremendous advances which have been made in medical knowledge through the study of conduction in the heart muscle we must the more lament our almost complete ignorance as regards conduction in the intestine.

Schillbach (2) thought the contractions resulting from electrical stimulation of the bowel ran farther upwards than downwards. Bayliss and Starling (3) found it hard to show descending inhibition in the rabbit because it extended at most 2 to 3 cm. and was so fleeting that it did not alter the appearance of the tracing to any great extent. In no case in the rabbit did excitation spread more than 5 to 6 cm. upwards. They had difficulty in showing any spread of the stimulus in the cat's bowel unless they gave castor oil. In the dog, the descending impulses traveled farther than the ascending. In fact, they could

not show the ascending excitation unless they stimulated just aborally to the recording balloon. On the other hand, they sometimes obtained effects two or three feet below the point stimulated (4). Conduction was slow—about 10 cm. per second. They had difficulty in estimating this rate on account of the long and variable latent period. After painting the bowel with cocain or after injecting the animal with nicotine the waves seemed to run equally well in either direction at a rate of 2.3 cm. per second. Stimuli evoked little response in these poisoned bowels.

There are a number of observations in other fields which would lead us to expect a better conduction in the aboral than in the oral direction. Child (5) has shown in many tissues that there is a constant stream of inhibiting influences descending along the gradients of metabolism which he has demonstrated. Tashiro (6) has explained the polarity of nerves on the same basis. The gradient of CO_2 production descends peripherally in motor nerves and descends centrally in sensory nerves. MacArthur and Jones (7) have shown a gradient of oxygen consumption in the central nervous system descending from the brain to the end of the cord. When angle worms (8), planarians and centipedes (9) are cut in two, the forward end may crawl on undisturbed while the hind end writhes convulsively. One explanation of this phenomenon is that conduction is almost entirely in the aboral direction. Carlson (9) has shown in the myriapoda that conduction along the ventral nerve cord is more rapid in the aboral than in the oral direction. This is true also for the spinal cord of the hag-fish (10) and probably for the cord of the snake (11). Of course in the spinal cord this difference might perhaps be due to a greater length and simplicity of the downward conducting paths. Sherrington has shown with the spinal cord of higher animals that inhibition spreads downward more easily than upward. In the decerebrate cat it is much easier to obtain reflexes in the hind limbs by touching the head than to get movements of the head or fore-limbs after touching the tail. "The exclusively aboral direction taken by shock seems to be universal in the nervous system" (12). It is suggestive that in the cord the region just above an injury seems often to be in a stimulated and hyperesthetic state much as the corresponding segment should be in the bowel.

The simplest nerve nets such as those in the medusae and sea-urchins conduct stimuli equally well in all directions (13). Others, however, like those in the feet of snails are "polarized" so that they conduct best in one direction (14). Parker (15) has suggested that the direc-

tion of peristalsis may be due to such a polarization of Auerbach's plexus.

Technic. Most of the work has been done on the small intestine of the rabbit because its contractions are so even and regular that abrupt changes in the record can with considerable certainty be interpreted as the effects of the stimuli used. Many experiments have been done also with the bowel of the cat. The rat's intestine was so insensitive to stimuli that we made only a few attempts to use it.

In order to simplify the problem we first studied the conduction in excised loops of bowel contracting rhythmically in warm aerated Locke's solution. Such a loop is fastened to the bottom of the vessel by serrefines which grasp it at two points 3 cm. distant from the ends. These short end segments are then turned up like the vertical arms of a U and connected to heart levers by means of threads. By turning up the recording ends of the loop in this way, one can avoid the use of pulleys.

In order to get a longer stretch of bowel between the recording ends and to have the bowel more accessible for the various types of stimulation, we replaced the usual tall narrow beaker with a long, comparatively shallow, glass bread-baking dish. On the bottom of this dish was a strip of cigar-box wood held down with lead weights. A centimeter scale was marked on its upper surface and at one end of the scale was fastened a little wire serrefine. At the other end was nailed a wooden upright with a little ring at its base and a cleat at the top. The segments were fastened in this apparatus as shown in figure 1. The water-bath surrounding the glass dish was kept at 38°C.

In some experiments the bowel was stimulated by pinching or by applying a crystal of NaCl. The most convenient form of stimulation and the only measurable one was the faradic shock. A convenient electrode for underwater work was made by inserting the wires from the secondary coil into two L tubes the lower ends of which were fastened on a short bar so that the bowel would just fill the space left between them. (See fig. 1.) A few experiments showed that the current kept very closely to the short path between the two tubes. A Harvard induction coil was used with one dry cell supplying 20 amperes at 1.7 volts. A tetanizing current was used.

In most of the tracings the movements recorded are those of the longitudinal muscle. When the serrefines were placed so that the circular muscle was recording, the amplitude of contraction naturally was less; the tendency to beat rhythmically was less and the irritability

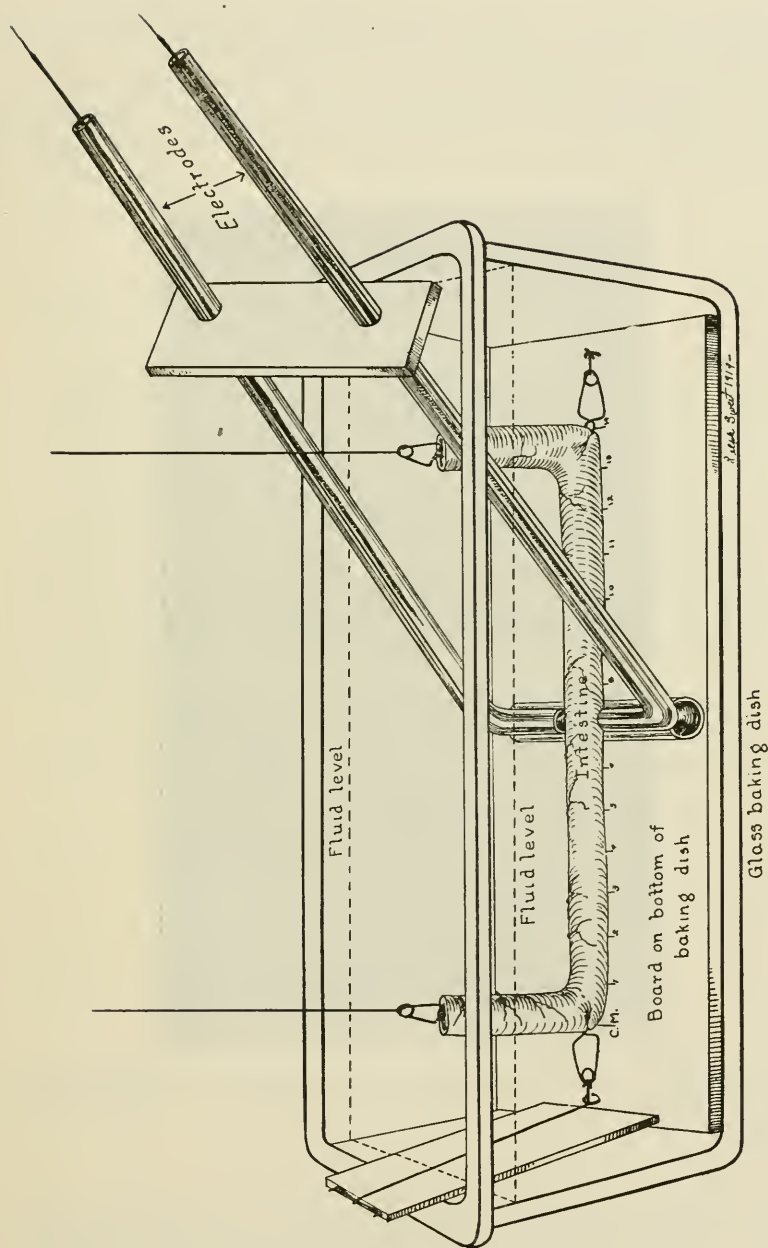


Fig. 1. Apparatus used in studying excised segments. For details see the text.

was often very low. Otherwise the results of stimulation were about the same as those obtained with the longitudinal muscle.

The work on the excised segments was repeated later on the intact bowel. The animals were anesthetized with urethane (2 gm. per kilo by mouth) and the cord was destroyed below the 4th dorsal vertebra.

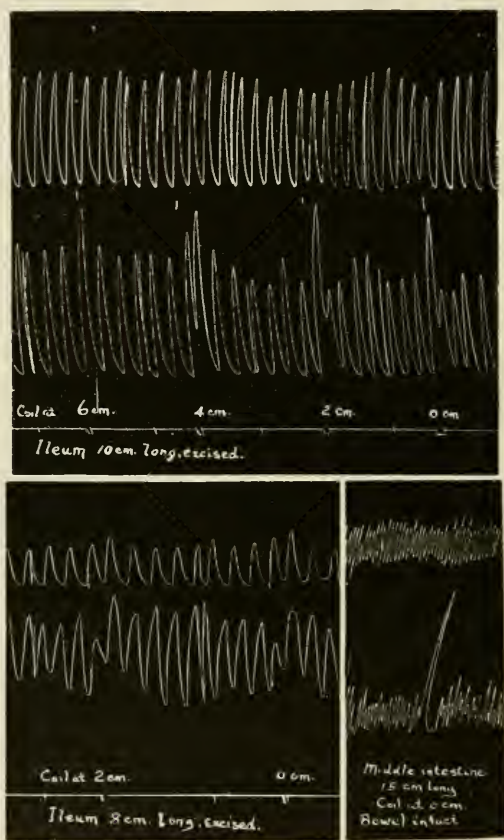


Fig. 2. When the bowel is stimulated midway between the recording segments the effects are most marked on the aboral end. The upper tracing is from the orad end.

The abdomen was opened under a bath of Locke's solution kept at 38°C. The best records were obtained with a very simple apparatus. Two glass rods were held vertically with their lower ends in the animal's abdomen. Fastened to the ends were little serrefines which seized the bowel at two points from 5 to 50 cm. apart, and held it down. Other

serrefines, attached by threads to the levers, were so arranged that two segments, 3 cm. long, and the desired distance apart, recorded their contractions on the drum. In some experiments we used the enterograph designed by Alvarez (16); in others we used balloons.

Conduction better in the aboral direction. The conclusions reached in this paper are based upon an analysis of over 2200 reactions. There were about 1000 each on the excised and intact intestines; 1700 were the results of electric stimuli; 400 the results of pinches and cuts, and the rest were the results of stimulation by salt crystals and balloons. In practically every instance it was easy to show that conduction is better in the caudal direction. If the distance between recorders was small enough so that a stimulus in the middle affected both tracings, the disturbance was more marked in the lower one than in the upper. (See fig. 2.) With a longer distance between the two, the lower recording segment would respond well to stimulation at the base of the upper when the upper failed to respond to stimulation at the base of the lower. (See fig. 3.) In other experiments the stimulating electrodes were brought closer and closer to the recording segment until it showed some response. The following table shows some of the results obtained in this way with strong faradic stimuli. It will be noticed that in one case a response was obtained 57 cm. below the point stimulated and only 7 cm. above.

DUODENUM		JEJUNUM		MIDDLE		ILEUM	
Orad	Caudad	Orad	Caudad	Orad	Caudad	Orad	Caudad
Excised segments							
5.5	12.5	5.5	15.0				
6.0	12.5	8.0	20.0	6.0	12.5	6.0	12.5
		6.0	15.0	6.0	15.0	6.0	13.0
10.0	18.0	6.0	15.0	6.0	15.0		
7.0	12. +	8.0	12. +	7.0	12. +		
Intact animal							
2.5	19.5			2.5	14.5	2.5	10.0
		9.5	19.5	9.5	48.0	7.0	14.5
		5.0	19.0	5.0	19.0	5.0	20.0
				4.5	36.0	4.5	36.0
		5.0	20.0			5.0	24.0
				5.0	43.0		
		9.5	43.0	10.0	43.0	12.0	72.0
				7.0	57.0		

The figures on any horizontal line are from one rabbit.

These differences were observed also with the mechanical and chemical stimuli, but they were not so striking. The experiments in which only the hind end of a worm reacts to a cut can sometimes be duplicated with a short U-loop of excised bowel.

The myenteric reflex. It will be noticed in the tracings that the characteristic response not only at the point stimulated but in the regions proximal and distal to it is an increase in amplitude and tone followed perhaps by a drop in amplitude and tone. In all this work we have seen very few examples of what might be called a "myenteric reflex." Thinking that perhaps this was due to the fact that we were using the longitudinal muscle when previous workers had used the

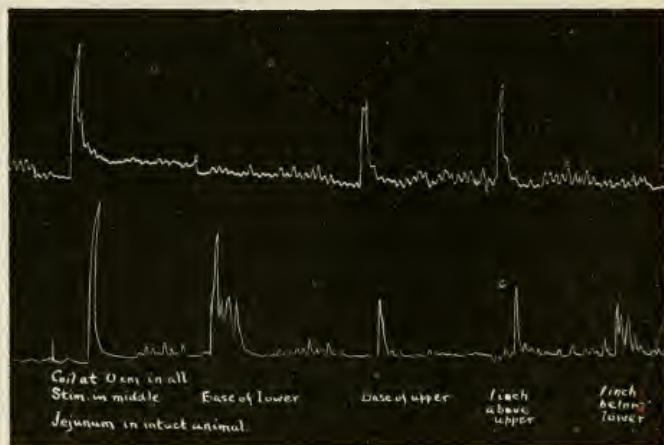


Fig. 3. When the bowel is stimulated above it responds below; when stimulated below it often fails to respond above. The upper tracing is from the oral segment. The distance between the recording segments was 5 cm.

circular we repeated the work attaching the serrefines so they would pull at right angles to the long axis of the bowel. In some experiments also we used balloons as others have done. With the excised segments the circular coat was slow to start beating and was often very unresponsive to stimuli. Otherwise the results were usually the same as those obtained with the longitudinal muscle. The greatest number of typical myenteric reflexes were obtained while using balloons—suddenly inflated and deflated—as the stimulus. We were not entirely satisfied, however, with the results obtained with these balloons. The trauma attendant upon their insertion and the violent

efforts of the bowel to force them out make conditions far from normal. It may easily be that the "myenteric reflex" is primarily a response to stretching and not a response to other forms of stimulation of the gut.

Observations on different animals. The rabbit's colon was too insensitive for satisfactory work. We had the same trouble with the excised small bowel of dogs and white rats. Considerable work was done with cats. The excised small intestine beat irregularly and was often insensitive. We were able to show, however, the same peculiarities of conduction that were seen in the rabbit. One cat was decerebrated so that we could avoid the use of urethane. As our results with this animal were the same as with others, the anesthetic probably had no appreciable effect on the bowel.

Remarkable differences were found in the irritability and conductivity in different animals of the same species. Previous work makes us feel that the degree of infection with intestinal and other parasites must have a good deal to do with these differences. Often the bowel was very insensitive in spite of a good rhythmicity.

Both with the excised segments and intact animals conduction seemed better in the middle region of the small intestine than at the ends. Poor results in the duodenum and upper jejunum could easily be explained, however, by the greater reaction to trauma and handling in that region. Sometimes the duodenum would respond well only to the first stimulus.

Rhythmicity. We had occasion to confirm the previous observation of Alvarez (17) that the rate of the oral end of an excised segment of rabbit's intestine is higher than the rate of the lower end. Keith (18) has suggested, on the basis of his anatomical studies and some of Alvarez's observations, that there are a number of rhythmic centers in the bowel, containing nodal tissue and dominating the rhythm of the adjacent regions. We have not been able to show any such dominance anywhere except occasionally in the terminal ileum (17). When the aboral recording end of a segment was severed from the rest of that segment its rate of contraction was never altered to any extent. There seem to be no such descending influences affecting the rhythm as there are in the heart. It is an interesting point, however, that when the longer segments were put into the aerated Locke's solution the lower end usually started contracting some time before the upper. This was due probably to a greater reaction to trauma caudad to the upper cut end than oral to the lower cut end.

Rate of conduction. Theoretically, an impulse should not only travel

farther with the gradient but it should travel faster with it than against it. We have made many attempts to show this but so far have not been able to obtain trustworthy records. The normal bowel is constantly in motion so that no satisfactory base line can be maintained. The muscle is slow in its reactions so that it is hard to say just when it begins to shorten. When it is contracting rhythmically a stimulus may show itself only as an increase in the height of the succeeding wave. Sometimes there is a temporary inhibition which delays the appearance of the reinforced wave. Still stronger inhibition may show itself as a drop in tone before the rise. But even when a fairly sharp take-off can be marked on the tracing the variations in latent period are so large that they may cover up differences due to the conduction time. Moreover, the latent period at a distance from the point stimulated may be lengthened considerably because the stimulus has become weakened during conduction. The different types of response to stimuli are shown in figure 4. A good deal of work was done with segments in which the rhythmic contractions had been more or less stilled by strychnin, adrenalin, nicotin, cocain and digitalis. Segments were studied also in baths of physiologic NaCl solution where they did not beat. Although some fairly satisfactory records were obtained in this way, slight tonus changes generally persisted even when the segments were so badly poisoned that their irritability and conductivity were practically gone. The most conclusive data were obtained from records of actively contracting untreated segments, stimulated half way between the two ends. Ordinarily, on account of the difference in rate at the two ends of the strip, one lever would be going up while the other was coming down, but occasionally for a few seconds the two ends would beat practically in unison. If stimuli were thrown in at those times it could often be shown that a fairly abrupt alteration in the tracing appeared first at the distal end. Following are some of the time intervals obtained. The segments were from 8 to 15 cm. long. The figures represent seconds elapsed after stimulation in the middle.

Excised segments

Upper end.....	2.0	1.5	4.2	4.2
Lower end.....	1.2	0.5	2.7	1.5

Intact animal

Upper end.....	0.8	1.3	1.1
Lower end.....	0.25	1.0	0.6

The conductivity varied markedly in the different animals. In two of the rabbits the impulse travelled 40 or 50 cm. in less than one second. Ordinarily the conduction time seemed to be much shorter. Following are some of the estimates made: 9, 15, 40, 27, 7, 10, 8, 30, 50, 20, 11,

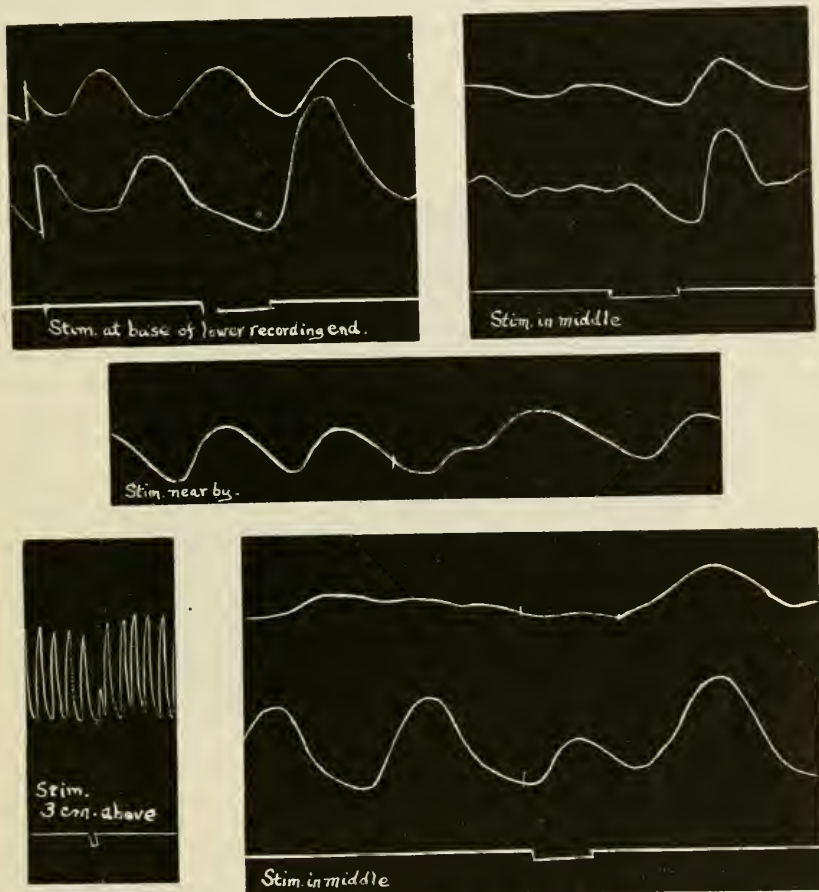


Fig. 4. To show different types of response to stimulation and the difficulties in the way of estimating the conduction time. The upper tracing is from the orad segment.

26, 16, 12, 40, 13, 22, 30, 11, 12, 40, 15. The average is 21 cm. per second. This figure would probably be higher with a more accurate technic. It represents the aboral rate. Conduction orad is harder to

measure as it is ordinarily limited to short distances where the error is larger.

At first sight the slow rate suggests conduction through the muscle, but a review of the literature on nerve-net muscle combinations shows that the impulses generally travel through the net and that the rate is usually slow. Thus the rate in *Cassiopea* is 27 to 50 cm. per second at 25°C.; in *Metridium* at 21°C. it varies between 12 and 14 cm. per second (19). The nerve net not only serves to expedite conduction but it keeps the muscle from contracting down hard. Several observers have noticed that when smooth muscle is cut off from its nervous connections its tone rises to a point where rhythmic contraction is impossible (20). It may be that some of the contractions in spasmodic ileus, in infantile pyloric stenosis and in Hirschprung's disease are due not to some abnormal stimulation but to the loss of this normal inhibition.

Some evidence was obtained at times of a more rapid conduction, perhaps by way of the mesenteric nerves. On two occasions sharp reactions were observed in the lower ileum two seconds after a stimulus had been applied to the greater curvature of the stomach. The distance along the bowel was from 300 to 400 cm. Frequently marked changes in the tone and rhythmicity of the loop studied would follow almost immediately after defecation, after small peristaltic rushes, or after handling the bowel 200 cm. or more above the region observed. The tone of the whole small intestine seemed to rise during efforts at defecation and it fell suddenly when food passed through the ileo-cecal sphincter. Figure 5 shows some of these long distance reactions. Bayliss and Starling have well said that "every point of the intestine is in a state of activity which can be played upon and modified by impulses arriving at it from all portions of the gut above and below" (21).

The rush waves along the bowel in the rabbit travel about 4 cm. per second. Theoretically they should go faster in the duodenum where the gradient is steepest and the metabolic rate fastest. They certainly seem to do so in man where the resultant rapid emptying accounts for the term "jejunum." In the long bowel of the rabbit a rush wave seems often to gain headway the farther it goes so that a wave which travels 2 cm. per second in the duodenum travels 6 cm. per second in the lower ileum.

No difference in reaction with strychnin. The work of pharmacologists suggests strongly that strychnin acts mainly upon the synapses between the neurones; it makes conduction across them easier and

therefore heightens reflexes (22). It may be then that we can follow Parker's suggestion (15) and use strychnin as an index to the simplicity or complexity of the nervous system in different animals. The simpler forms of life with pure nerve nets should show only the toxic protoplasmic effects of strychnin, while the higher forms with more and more synapses should show heightened reflexes and clonic spasms. Moreover, we might find that disturbance in the mechanism of reciprocal innervation which has been observed not only in the spinal cord of mammals but even in earthworms and starfish after the administration of strychnin (23). Although considerable work must yet be

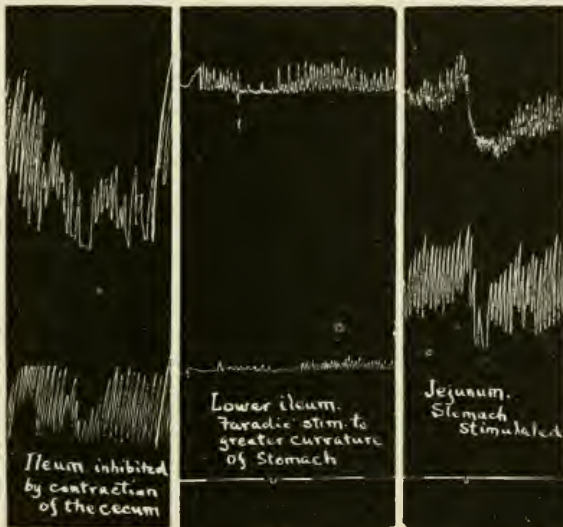


Fig. 5. Intact bowel affected by activity of the cecum and by stimulation of the greater curvature of the stomach.

done before we can say how dependable this strychnin test is, it is certainly suggestive that we have been unable to show much difference between the conduction time and the type of reaction to stimuli in strychninized and normal animals.

We kept a number of our rabbits and cats for several hours under large and repeated doses of strychnin. At no time could we show any improvement in conduction or any change in the type of reaction to stimuli, even when the animals were twitching and going into convulsions. (It should be remembered that they were under urethane

anesthesia.) Similar experiments were performed with the excised segments, beating in Locke's solution to which strychnin had been added. Frequently conduction was decidedly impaired by the drug but almost always it remained better in the caudad than in the orad direction. Auerbach's plexus then would appear to be a simple net, without reflex arcs and without synapses excepting those between the net and the central nervous system. This conclusion agrees with that of the anatomists who, after much argument and research, have finally decided that there are no commissural fibers in the involuntary nervous system such as would be necessary for the working of true reflexes (24).

SUMMARY

It has been shown that there is a metabolic gradient in the small intestine from duodenum to ileum. As would be expected, conduction is better with the gradient than against it.

The rate of conduction could not be determined accurately. It appears to be about 20 cm. per second. At times it is about 150 cm. per second, probably by way of the nerves in the mesentery.

In the rabbit, the peristaltic rushes travel about 4 cm. per second.

The characteristic response to a stimulus applied to the gut is a contraction above and below. This may be preceded or followed by an inhibitory phase. The "myenteric reflex" was rarely observed, and then usually after distension by balloons.

The tone and activity of any part of the tract can be affected markedly by the activities of other parts.

With the exception of the terminal ileum, no part of the bowel seems to affect the rate of rhythmic contraction of adjacent parts. This finding is against the theory of peristalsis offered by Keith.

The failure of strychnin to influence conduction or to alter the type of response to stimuli suggests that Auerbach's plexus is a simple nerve-net, without synapses or reflex arcs.

BIBLIOGRAPHY

- (1) ALVAREZ AND STARKWEATHER: This Journal, 1918, xlv, 186.
- (2) SCHILLBACH: Arch. f. Path. Anat. u. Physiol., 1887, cix, 281.
- (3) BAYLISS AND STARLING: Journ. Physiol., 1900, xxvi, 116, 128, 134, 110.
- (4) BAYLISS AND STARLING: Ibid., 1899, xxiv, 113, 115.
- (5) CHILD: Senescence and rejuvenescence, Chicago, 1915.
- (6) TASHIRO: A chemical sign of life, Chicago, 1917.
- (7) MACARTHUR AND JONES: Journ. Biol. Chem., 1917, xxxii, 259

- (8) FRIEDLÄNDER: *Biol. Centralbl.*, 1888, viii, 363.
NORMAN: *Arch. f. d. gesamt. Physiol.*, 1897, lxvii, 137.
- (9) CARLSON: *Journ. Exper. Zool.*, 1904, i, 269.
- (10) CARLSON: *This Journal*, 1903, x, 401.
- (11) CARLSON: *Arch. f. d. gesamt. Physiol.*, 1904, ci, 233.
- (12) SHERRINGTON: *Schäfer's Handbook of physiology*, 1900, ii, 822, 833, 846;
The integrative action of the nervous system, London, 1911, 163.
McGUIGAN, KEETON AND SLOAN: *Journ. Pharm. Exper. Therap.*, 1916, viii, 143.
- (13) BETHE: *Allg. Anat. u. Physiol. d. Nervensystems*, 1903.
- (14) BIEDERMANN: *Arch. f. d. gesamt. Physiol.*, 1906, cxi, 260.
- (15) PARKER: *Science*, 1918, xlvii, 151.
- (16) ALVAREZ: *This Journal*, 1915, xxxvii, 271.
- (17) ALVAREZ: *Ibid.*, 1914, xxxv, 177.
- (18) KEITH: *Lancet*, 1915, ii, 371.
- (19) HECHT: *This Journal*, 1918, xlv, 157.
PARKER: *Journ. Gen. Physiol.*, 1918, i, 231.
JENKINS AND CARLSON: *This Journal*, 1903, viii, 251.
- (20) BIEDERMANN: *Arch. f. d. gesamt. Physiol.*, 1905, cvii, 43.
BETHE: *Allg. Anat. u. Physiol. d. Nervensystems*, 1903.
- (21) BAYLISS AND STARLING: *Journ. Physiol.*, 1899, xxiv, 116.
- (22) PORTER: *This Journal*, 1915, xxxvi, 171.
McGUIGAN, KEETON AND SLOAN: *Journ. Pharm. Exper. Therap.*, 1916, viii, 143.
- (23) MOORE: *Journ. Gen. Physiol.*, 1918, i, 97.
KNOWLTON AND MOORE: *This Journal*, 1917, xlv, 490.
MOORE: *Journ. Pharm. Exper. Therap.*, 1916, ix, 167.
- (24) GASKELL: *The involuntary nervous system*, London, 1916.
LANGLEY: *Journ. Physiol.*, 1904, xxxi, 244.
CARPENTER AND CONEL: *Journ. Comp. Neurol.*, 1914, xxiv, 269.
JOHNSON: *Ibid.*, 1918, xxix, 385.

THE RADIOGRAPHIC STUDY OF THE ABDOMINAL ORGANS AFTER INFLATION OF THE PERI- TONEAL CAVITY.*

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As is well known, any part of the body may be made visible under the Roentgen rays if we can only make its degree of resistance to the passage of those rays different from that of the surrounding tissues. We may make it more resistant by injecting suspensions of barium or we may make it less resistant by injecting air. Many have used air or oxygen in radiographing the bladder, colon (1), stomach (2), and knee-joints (3). Recently oxygen has been used to outline the cerebral sinuses. For a number of years the German radiologists have been experimenting with the injection of air or oxygen into the peritoneal cavity. Lorey (4) in 1912 seems to have been the first to show radiographs taken after injecting air into the abdomen of a patient who had been tapped for ascites. A great deal of credit should be given to Weber (5) who began in 1912 to work out this technic on animals and cadavers. His published plates are excellent and it is surprising that his epoch-making article in a widely read journal should have been so completely ignored and forgotten by the profession. Although a few papers (6) appeared on the subject in the next few years, it was not until 1918 (7) that the men in Europe seemed to wake up to the possibilities of this method of diagnosis. While in the East last June I saw some beautiful plates taken with this method by Drs. Stein and Stewart of New York (8). These impressed me so much that immediately upon my return to San Francisco I began ex-

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- (1) Henzelmann: Wien. klin. Wchnschr., 1918, 31, 915.
Löffler: Münch. med. Wchnschr., 1914, 61, 763.
- (2) Niden: Deutsche med. Wchnschr., 1911, 37, 1515.
- (3) Hoffa: Berl. klin. Wchnschr., 1906, 43, 940.
- (4) Jacobsohn: Deutsche med. Wchnschr., 1907, 33, 703.
- (5) Lorey: Verhandl. d. dtsch. Roentgen Gesellsch., 1912, 8, 52.
- (6) Weber: Fortschritte a. d. Gebiete d. Roentgenstr., 1913, 20, 453.
- (7) Meyer-Betz: Münch. med. Wchnschr., 1914, 61, 810.
Rautenberg: Dtsch. med. Wchnschr., 1914, 40, 1205.
Berl. klin. Wchnschr., 1914, 51, 1608.
- (8) Goetze: Münch. med. Wchnschr., 1918, 65, 1275.
Schmidt: Deutsche med. Wchnschr., 1919, 45, 201.
Schittenhelm: Deutsche med. Wchnschr., 1919, 45, 566.
Rautenberg: Berl. klin. Wchnschr., 1917, 54, 22.
Alessandrini: Policlinico, 1919, 26, 641.
- (8) Stein and Stewart: Ann. Surg., 1919, 70, 95.

perimenting on animals to satisfy myself of the harmlessness of the procedure, and later to see if I could modify it so that it would be more convenient for use at the office. Its harmlessness in suitable cases seems to have been well established, as no accidents have been reported from the clinics in which it has been used extensively. Rabbits and guinea pigs can be distended with O_2 or CO_2 to a degree not approachable in man, without producing any signs of distress or concern. Strange to say the rapid absorption of these large quantities of gas does not bother their respiratory centers.

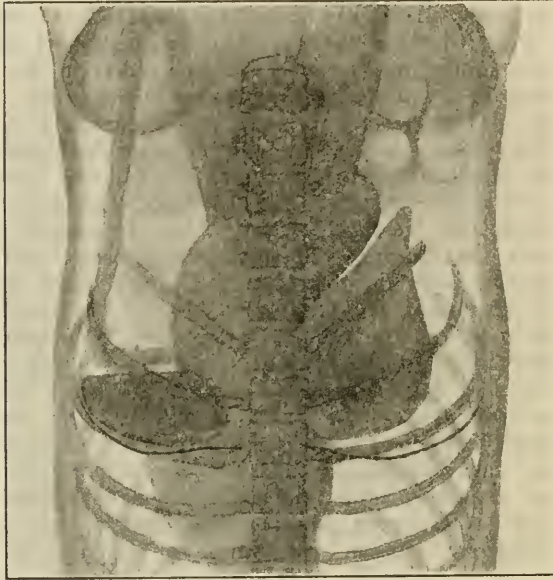


FIGURE 1.—Taken with the patient lying prone. Note the heart, lower ribs, diaphragm; spleen, liver, kidneys, descending colon containing traces of barium; loops of small bowel; cecum and ascending colon full of gas; ilio-psoas muscles and pelvic brim.

The more I work with this method on man the more enthusiastic I become, and the more convinced that we have here the biggest advance in radiologic technic since the introduction of the bismuth meal by Cannon in 1898.

Technic.

The patient ordinarily should have the bowel and stomach empty. In certain cases, however, good results are obtained by having the bowel filled with barium. I believe it advisable to give the patients a quarter of a grain of morphin hypodermically fifteen minutes before the injection because otherwise some will be very restless and will complain of pain. Nervous women will be quieted by the sedative, and will be less likely to get panicky if they feel faint and oppressed about the heart. Some of the more phlegmatic in-

dividuals do not seem to feel much distress and complain only about the abdominal distension.

I use a spinal puncture needle which is thrust through the left rectus muscle near the navel. The skin is painted with a little iodine. No anaesthetic is needed. With a little practice one can tell when the point of the needle goes through the peritoneum. I generally inject first a little sterile normal salt solution to make sure that the needle is clear and properly placed. It then is connected with a small rubber bag which contains a liter or two of gas. There appears to be no need for sterilizing or washing this gas. Experiments on animals and all the experience on man indicate that, with ordinary care, there is no danger of damaging the bowel with the needle. The amount of gas injected depends on the feelings of the patient. A compromise often has to be made between the desire of the radiographer to get good plates by the injection of large amounts and the desire of the patient to be let off easy. When enough has been introduced, the needle is removed and the work of taking plates is begun. If possible the patients should be kept lying down until their gas has been absorbed or until they are comfortable again. When they sit up, the pain in the shoulders becomes acute and they may feel as if they were going to collapse. Strange to say, after an hour or two they may be able to walk around quite comfortably, although it can be seen with the

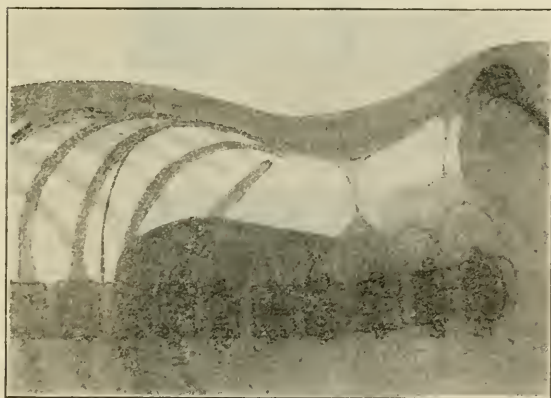


FIGURE 2.—Taken with the patient on the left side. Note the lower ribs, upper surface of the liver; and peritoneal bands running to a prolapsed kidney lying transversely across the spine, and to coils of intestine.

screen that the liver is four inches or more below the diaphragm. Very satisfactory plates can be taken with the patient prone. Particularly when the gall-bladder region is raised a little on the inclined plane which is used in taking frontal sinuses, the liver and kidney shadows are separated and beautifully distinct. The gall-bladder can be shown better with this technic than with any other, and there no longer is any need for catheterizing the right ureter when we want to

tell whether a stone shadow is in the gall-bladder or in the pelvis of the kidney. Adhesions to the gall-bladder may also be shown very clearly.

Stereoscopic plates taken in the prone position are beautiful, and will show the liver, spleen and kidneys in all their outlines as clearly as if they were exposed by dissection. With the patient first on one side and then on the other, the diaphragm stands out as a thin line on the plate. The lower ribs show so clearly that no difficulty need be encountered in showing old fracture lines. The upper surface of the liver can be seen perfectly so that adhesions, cirrhotic deformities and syphilitic or carcinomatous nodules can be recognized. Peritoneal adhesions, tumors of the colon and small amounts of ascitic fluid may also be seen. The stomach may be inflated a little, where-

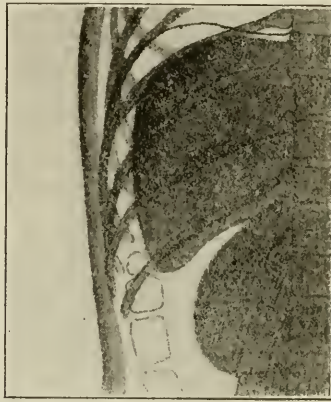


FIGURE 3.—Taken with the patient lying prone. Shows the diaphragm; normal liver and kidney and the haustra of the ascending colon filled with gas.



FIGURE 4.—In comparison with the preceding plate, note the enlarged and thickened gall-bladder and the coils of bowel which do not drop down out of the way. Operation showed a large, thickened gall-bladder without stones. There were strong bands of adhesions binding the hepatic flexure to the hilum of the liver.

upon its wall can be studied. The relations of tumors to surrounding organs may become clear. In the Trendelenburg position the uterus and even the ovaries may be visualized. In one of Dr.

Stein's plates one can see cysts in the ovaries. Adhesions to the anterior abdominal wall can be shown by taking a lateral plate with the patient on his back. Lesions of the spine and aneurisms of the abdominal aorta can be demonstrated clearly when they are present. Occasionally one can see calcified mesenteric glands.

An Improvement in the Technic.

After working with this technic for a few days it seemed to me that its usefulness would have to remain limited largely to hospital patients unless some means could be found of getting rid of the gas more promptly. Although in most cases the oxygen was sufficiently absorbed in two or three hours so that the patient could leave the office, few cared to work next day; and one man still had a large amount of gas in his abdomen after five days. Hence Dr. F. B. Taylor and I began injecting rabbits at the Hooper Foundation with various gases and soon found, as we expected, that CO_2 would be absorbed many times faster than O_2 . After satisfying ourselves that the procedure was harmless, we began using CO_2 at the office and have since made it almost a routine. The great advantage of this method is that we can assure the patient that in twenty-five minutes his gas will be out, his distress will be over, and he can go back to his work as if nothing had happened. The disadvantage is that the operator must work rapidly if he is going to get all the plates he wants. Moreover, if a plate should be unsatisfactory for any reason, or if on development, something should be found which requires further study, it may be too late. We overcome this difficulty somewhat by having three people working rapidly; one developing as fast as the plates are taken. It may be, now, that by adding a little O_2 we can slow the emptying a little and yet retain the great advantages which have been gained with the new technic.

Summary.

A technic is described which the writer believes marks the biggest step in advance as regards intra-abdominal diagnosis since the bismuth meal was introduced.

After injecting gases into the peritoneal cavity the intestines will float out of the way and the various organs will move around so that beautiful X-ray plates can be secured of the diaphragm, liver, spleen, gall-bladder, kidneys, gastro-intestinal tract, spine, uterus and ovaries.

This technic has proven particularly helpful in the diagnosis of gall-bladder disease.

By using CO_2 instead of O_2 the writer has modified the original procedure so that it may now be used in the office as well as in the hospital. Whereas the O_2 leaves the abdomen in from 24 to 100 hours, the CO_2 leaves in half an hour.

Note—Inasmuch as it is impossible to reproduce the finer details of radiographs on ordinary printing paper, I have had drawings made from a few of my plates. Those who saw these plates at a recent meeting of the San Francisco County Medical Society will, I think, agree with me that Mr. Ralph Sweet, the medical illustrator at the University of California, has copied them very faithfully.

BLOOD PRESSURE IN UNIVERSITY FRESHMEN AND OFFICE PATIENTS *

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SAN FRANCISCO

What is the average blood pressure for a man or woman of a given age? How common is hypertension in young people? What is its significance, particularly in early life? These are some of the questions which occurred to me after taking the blood pressures on 265 men called in the closing days of the second draft.¹ Although clinical experience had led me to expect a high incidence of hypertension in young men, it had not prepared me for the finding that in this particular series there were more with pressures over 130 mm. than under; and very many with pressures between 160 and 275. It was soon clear that this group did not represent a fair sampling from the community because so many were rejects from the recruiting offices; that is, most of the physically fit had gone to war and the unfit were left.

On turning to the available statistics, it was surprising to see that most of them must be objected to on this same score of previous selection. Thus Smith² studied 500 aviation recruits, Sorapure³ 796 soldiers, Goepp⁴ 9,996 accepted insurance cases, Fisher⁵ 12,647 accepted insurance cases, MacKenzie⁶ 31,934 accepted insurance cases, and Woley⁷ 1,000 insurance cases which remained after excluding those with histories or physical findings suggestive of cardiovascular abnormality. The insurance statistics would have much more value if they were based on the examination of all applicants; but even then there would undoubtedly be a considerable amount of adverse selection because those with hypertension often have premonitions of future illness; and many seek insurance after discovering their defects.

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1. Alvarez: California State J. M. **17**:367, 1919.

2. Smith, B.: J. A. M. A. **71**:171 (July 20) 1918.

3. Sorapure: Lancet **2**:841, 1918.

4. Goepp: Penn. M. J. **22**:295, 1919.

5. Fisher: Proc. Assoc. Life Ins. Med. Directors of America; N. Y., 394, 1912; 90 and 246, 1915; 203, 1917.

6. MacKenzie: Ibid, 221, 1917.

7. Woley: J. A. M. A. **55**:121 (July 5) 1910.

The next question then is: how are we to get a fair sampling? An ideal way would be to examine every tenth individual passing a certain point on a busy street. Different strata of society might be sampled by choosing streets in different parts of the city and also by choosing different cities. Unfortunately, such methods are not practicable at present. The next best thing probably would be to study the young people gathered together in the public schools and colleges. Particularly in state universities where tuition is free it would seem that the student body should represent a fair sampling from the more intelligent part of the community. Fortunately, it is becoming more and more the custom in the leading schools to examine the incoming freshmen. Lee⁸ and Barach and Marks⁹ have already reported on the blood pressure readings in some 1,200 college students, but their groups were not large enough for analysis by age and sex.

During the last few years all the freshmen entering the University of California have been subjected to a physical examination which includes the measurement of the blood pressure. Under ordinary conditions this mass of data which has accumulated at the university infirmary would have been ideal for my purposes. Unfortunately, however, the war with its demands on the fittest of the young men, has made me uncertain of the sampling even in the ages below 21. In 1918 most of the men were gone; but some were enrolled in the S. A. T. C., and some probably remained on account of physical handicaps which turned them back from the recruiting offices. Certain it is that there is a big difference between the average pressures of the male entrants in 1919 and the pressures of those in the three preceding years. As would be expected, there is practically no difference in the figures for the corresponding groups of women (Fig. 5). I hope to clear up these uncertainties by getting more and better data in the next few years.

TECHNIC

Unfortunately, the methods of taking the pressures are not exactly the same for men and women. The men take a tepid shower before the examination and are then examined in the reclining position. They are more likely to stand around in negligé and get cold than are the women. The women's pressures are taken standing. The systolic pressure, the only one studied at this time, was taken by palpation. This is the more reliable method as it is less subject to wide variations. One aneroid (a large Tycos) and three mercury column instruments were used. The aneroid was checked at regular intervals against a

8. Lee: Boston M. & S. J. **173**:541, 1915.

9. Barach and Marks: Arch. Int. Med. **13**:648 (May) 1914.

mercury column. The records were taken off the cards and arranged as in Tables 1 and 2. This shows, first, that the data on the women are much better for statistical purposes than those on the men because the women examiners have read quite closely to the millimeter, while the men have had a strong tendency to read to the nearest multiple of ten. For practical purposes, and especially when measuring a value that often fluctuates from hour to hour, it is of little importance whether a pressure is 150 or 160 mm., but for statistical work and especially for drawing curves of frequency distribution, it is much better to have the data evenly distributed. It is useless to try and make the steps less than 2 mm. because that is the unit on most of the scales.

A glance at Table 1 shows that the lowest age is 16. There were 99 women and 34 men who entered the university at that age during the period covered. Three thousand, three hundred and thirty-three (3,333) entered between the ages of 18 and 19. The curves of frequency distribution for the different ages for the two sexes are shown in Figure 1. The abscissae represent the blood pressures by 5 mm. Stages. In these units "85" = 85 to 89, and "90" = 90 to 94. When 85 is made to include from 83 to 87 the only difference in the curves is one due to the exaggeration of the deformity brought about by the grouping of the data about the multiple of ten. The ordinates represent percentages at the different pressures.

A study of these curves shows several interesting things. In the first place, as Kilgore¹⁰ has pointed out, they are fairly symmetrical and correspond to the type "A" curve so well known to statisticians. It is the curve obtained when we chart the heights of a thousand men, the errors in 100 measurements of a meter bar, or the results of a thousand throws of dice. In all these studies of large groups of observation it has been found that most of the data will lie more or less closely about the average. Hence it is that the probability that small errors or deviations from the average will occur is larger than the probability that large errors or deviations will occur. Thus, in measuring a thousand men with an average height of 5 feet 6 inches, the probability of finding men 5 feet 5 inches, and 5 feet 7 inches in height is much greater than the probability of finding men 5 feet or 6 feet tall. There will be scores of the former to one or two of the latter. With the help of mathematical formulae we can calculate just how many of the data are likely to fall within certain limits measured on each side of the average. The divergences between the calculated and the actual curves are often very slight as will be noted in Figures

10. Kilgore: *Lancet* 2:236, 1918.

2 and 3. A study of the differences which do appear is sometimes very helpful.

The simplest and shortest way in which to compare the curves is to plot the data on paper which has been ruled according to the theory of probability, with small spaces near the 50 per cent. mark and wide ones near the 0.01 and 99.99 per cent. marks. If the data follow the theory, they will plot out in a straight line. Those who have difficulty in following this part of the discussion would be much helped by reading Whipple's¹¹ and Rugg's¹² little books on applied statistics.

Figure 3 shows the data for 5,807 women and 2,930 men plotted on probability paper. It will be noted that the figures for the women between pressures of 110 and 130 lie along a straight line; as do those for the men between 90 and 140 mm. This suggests strongly that within those limits deviations from the average are due to various small errors subject to the law of chance. Deviations above and below these limits are probably due to pathologic factors. It will be noted also that the line for the women is more nearly vertical than that for the men. This demonstrates a fact which will be shown again later in other ways, and that is that the dispersion is less for the women: i. e., their readings are grouped more closely about the average. This is well shown in Figure 2 where we can compare the distribution curve for the women with that for the men. The women's curve is high and narrow; the men's is low and wide. The average for the women is 115 mm.; for the men 126.5 mm. If we get the average of the deviations from the average we find that for the women it is 8.0 mm., and for the men 10.8 mm. In Figure 2 the distribution curves have been smoothed so as to remove the humps which are seen in Figure 4. This smoothing is done by averaging the values of different points on the curve with the values of the two adjacent points (Rugg,¹² p. 184). The solid lines represent these smoothed curves. The lines of dots and dashes represent the probable curves secured by plotting the intersections of the two straight lines drawn through the data in Figure 3. The dotted lines represent the theoretical curves obtained mathematically, using the so-called standard deviation which for the women is 2.12 (of the 5 mm. abscissae units) and for the men, 2.98. Given these data it is easy to calculate the values of the ordinates corresponding to the different abscissae. The results of the graphic and mathematical methods are so close for the women that only one theoretical curve has been plotted.

11. Whipple: *Vital Statistics*, N. Y., 1919.

12. Rugg: *Statistical Methods Applied to Education*, Boston, 1917.

TABLE 1.—BLOOD PRESSURE OBSERVATIONS ON WOMEN STUDENTS

Pressure	Ages												Percentage	Theoretical Percentage from Paper	Theoretical Percentage Computed from Standard Deviation	Smoothed Curve in Percentages
	16	17	18	19	20	21	22	23	24	25	27	31	36	40	Total	
80-84	1	2	1	4	0.07
85-89	2	14	0.31
90-94	1	8	9	5	1	9	3	1	32	0.55
95-99	4	13	34	23	12	13	12	7	8	7	10	7	157	1.13
100-104	4	34	111	83	68	39	36	28	18	32	41	24	19	514	320	3.20
105-109	19	62	104	110	64	55	38	31	18	97	56	35	14	633	11.30	6.50
110-114	14	95	281	223	118	122	61	49	56	61	75	67	25	1,247	23.00	17.00
115-119	12	74	161	145	109	122	40	47	40	61	54	45	21	879	32.00	19.00
120-124	20	94	221	188	103	97	60	45	26	52	72	57	23	1,058	18.20	17.30
125-129	19	65	135	108	54	39	24	11	16	18	27	22	11	403	9.80	8.73
130-134	5	45	89	63	41	31	13	3	3	5	5	12	5	157	6.94	7.10
135-139	6	15	36	30	17	3	7	4	1	2	1	2	2	92	2.70	3.20
140-144	1	10	22	17	2	4	1	98.30	3.30
145-149	3	99.55	1.25
150-154	1	99.90	0.35
155-159	99.98	0.08
160-164
165-169
Total.....	99	512	1,214	1,014	570	503	317	259	201	277	383	302	150	5,807	115.0	0.03
Average..	115.8	117.2	116.5	116.2	116.2	115.7	116.5	115.4	115.4	114.7	115.4	117.3	118.3	0.07

Average deviation of the 5,807 = $1.6 \times 5 = 8$ mm.

Standard deviation = $2.12 \times 5 = 10.6$ mm.

Coefficient of variation = $\frac{100 \times \text{S.D.}}{\text{M}} = \frac{212}{115} = 1.85$

TABLE 2.—BLOOD PRESSURE OBSERVATIONS ON MEN STUDENTS

Pressure	Ages												Total	Percentage	Theoretical Percentage from Probability Paper	Theoretical Percentage Computed from Standard Deviation	Smoothed Curve in Percentages
	16	17	18	19	20	21	22	23	24	25	27 to 30	31 to 35	36 to 40				
80-84	3	3	0.06	0.06	0.10
85-89	0.20	0.14	0.21
90-94	1	0.60	0.54	0.21
95-99	2	1.70	1.10	1.33
100-104	1	4.00	2.30	2.40
105-109	2	8.00	4.00	3.75
110-114	13	15.50	7.50	7.16
115-119	4	26.00	10.50	11.78
120-124	9	39.50	13.50	13.77
125-129	4	54.00	14.50	14.39
130-134	6	68.00	14.00	13.34
135-139	2	80.00	12.00	10.26
140-144	3	88.50	8.50	9.00
145-149	1	94.40	5.90	4.58
150-154	1	97.40	3.00	3.41
155-159	99.00	1.00	2.25
160-164	1	99.55	0.55	1.46
165-169	2	99.90	0.45	0.76
170-174	99.96	0.06	0.43
175-179	99.99	0.03	0.23
180	0.07
Total.....	34	212	560	545	362	330	233	178	113	139	117	66	40	2,930			
Average...	126.3	128.2	127.8	126.2	126.3	125.4	126.2	126.3	130.0	126.9	126.0	126.8	126.5			

Average deviation of the 2,930 = $2.17 \times 5 = 10.85$ mm.
Standard deviation = $2.98 \times 5 = 14.90$ mm.
 $100 \times \text{S.D.} = \frac{298}{298} = 2.35$
Coefficient of variation = $\frac{M}{126.5}$

A glance at these curves shows that the agreement between the found and the theoretical data is close. The main difference is that the actual curves drop off a little too rapidly at first on the upper side and then later they do not drop off fast enough. This deformity of the curves suggests strongly that the figures over 137 mm. for the women and over 148 mm. for the men represent pathologic conditions. If we turn again to Figure 3, we see that according to the theory of probabilities only one man in 10,000 should have a pressure over 175. Actually there were eight in 2,930 or twenty-seven in 10,000. Similarly, there should have been only one woman in 10,000 with a pressure over 150; actually there were twenty-five in 5,807 or 43 in 10,000.

The higher figures are modified by the incidence of hypertension; the lower ones by fainting and perhaps by convalescence from acute infections. I threw out a number of records below 90 mm. on which fainting was definitely noted, but it is likely that many of the low records included might also have been removed for this reason. To be entirely consistent, I should probably have left these records in, just as I have left in the high records in cases where excitement may have been a disturbing factor.

In Figure 1 it will be noted that the modes or apices of the men's curves lie between 120 and 130; those of the women lie between 110 and 120. This difference of 10 mm. between the pressures of the two sexes has been noted by previous observers. It is remarkable that there is practically no change in the location of the mode between the ages of 18 and 40. That for the men is actually a little lower at 33 than at 17; and that for the women is lower at 38 than at 16.

There are some differences in the frequency polygons for the various age groups, but owing to inaccuracies in the readings, to the smallness of some of the groups and to uncertainties about the sampling we cannot lay much stress on them. The sexual difference is most marked at the age of 24. It is suggestive that the curve for the 16 year old boys resembles that for the women; and that for the women over 36 resembles that for the men. The possible significance of this finding will be discussed later.

THE AVERAGE BLOOD PRESSURE

Figure 4 shows the arithmetical means (averages) of the different age groups plotted on coordinate paper. It will be seen that the curve of average pressure not only does not rise steadily as it has been supposed to do, but actually drops from 17 to 25 in the women and from 17 to 21 in the men. This finding was so unexpected that every care has been taken to insure the accuracy of the figures. Ordinarily, such averages are obtained by grouping the data and then applying

short-cut methods. In this case no grouping was done, and the figures charted represent the means of the actual data as they were taken off the records. As there was considerable doubt about the reliability of the sampling in the men's group, I divided the data into two parts: those obtained in 1919, and those obtained before that. Figure 5 shows the average for the two groups separately and combined. Many of the ages are not represented because the number of readings was too small. In order to save time these averages were computed by the short-cut method. The dotted line is that of the more exact averages shown in Figure 4. These curves show the reliability of the sampling for the women. The 1919 women (between 17 and 22) average about 1.0 mm. higher than do those for the preceding years. This difference is so small and so consistent that it may be due to the personal equation of the examiners or to errors in their instruments. The trend of the average in both groups of women is downward during these years.

When we turn to the curves for the men we find differences so large that it would seem that they must be due to disturbances in the sampling. This difference ranges from 9 mm. at 18, to 3.5 mm. at 23; the 1919 men showing the higher figures. The average of the 1919 men falls from 18 to 23, while that of the pre-nineteen men rises from 18 to 24. No significance can be attached to the big rise in the combined average at 24 because it is not present in the pre-nineteen curve, unless it be that the rise at 23 there corresponds to the rise at 24 in the 1919 curve. It is hard to explain the big differences in the men's curves as we do not know enough about the ways in which the sampling was modified. Some might perhaps ascribe the increase in average pressure in 1919 to the strain of the War year, but that seems to me improbable.

There is little doubt about the drop from 17 to 25 in the women's curve as it appears in both the 1919 and the preceding groups. It appears also in the curve drawn from data secured on 1,000 of my office patients. This drop does not seem to have been described heretofore. The most extensive studies of the yearly average have been made by MacKenzie⁶ and Fisher.⁵ Their figures, for men only, when plotted on coordinate paper, lie in a practically straight line from 119 mm. at 17 to 140 mm. at 66. Unfortunately, these observations have little value to us; the line had to be straight because only those men were accepted who varied within prescribed limits from a prescribed standard.

After 25, the average curve for the women rises rapidly. In the series of office patients it was found to rise so much more rapidly than that for the men that it crossed over about the age of 40. After

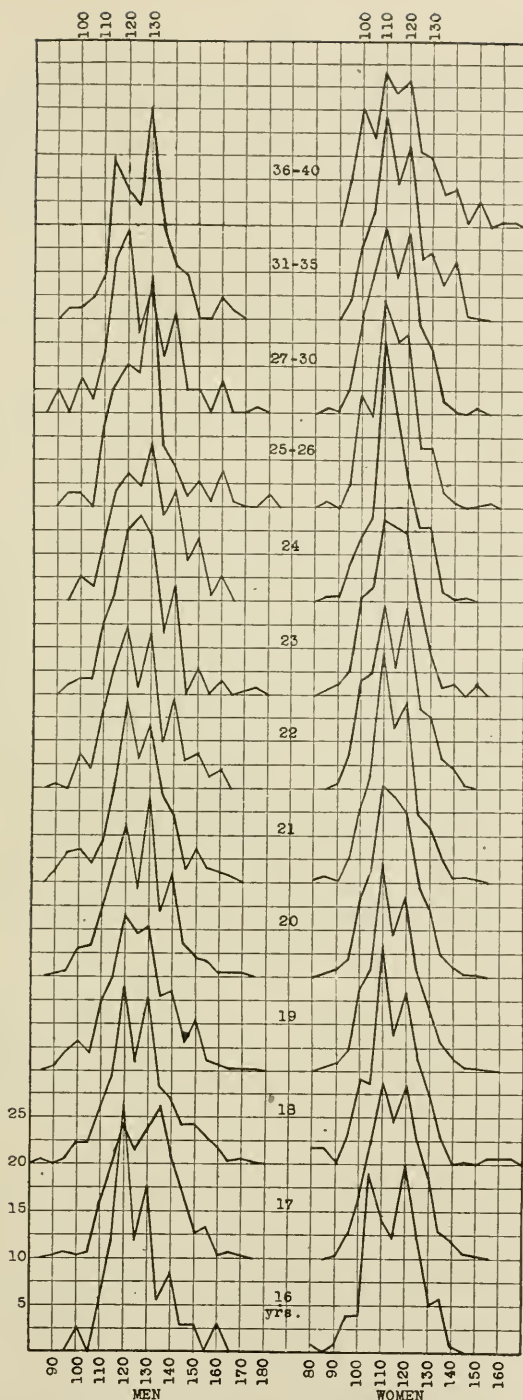


Fig. 1.—Frequency distribution curves for men and women students at different ages. The abscissae are pressures in mm. and the ordinates are percentages. In order to shorten the chart the ordinates for different age groups are made to overlap.

that the women averaged higher than the men (Fig. 6.). What we need now is more information about the trend of these average curves below 17 and above 35. We must know where the sexual difference appears and when it disappears or becomes reversed. Work is now being done on this problem in the children's clinic at the Universities of California and Stanford.

Another feature of the frequency distribution curves in Figure 1 which requires comment is their apparent bimodality, i. e., their tendency to show two peaks. Exceptions are found in the men at 16, 19, 23, 24 and 25, and in the women at 20, 23, 24, 25 and 36. This peculiarity is due in most instances to the tendency of the examiners to read to the nearest multiple of 10 on the scale. That tendency, however, will not explain the bimodality of the men's curves at 17 and 31.

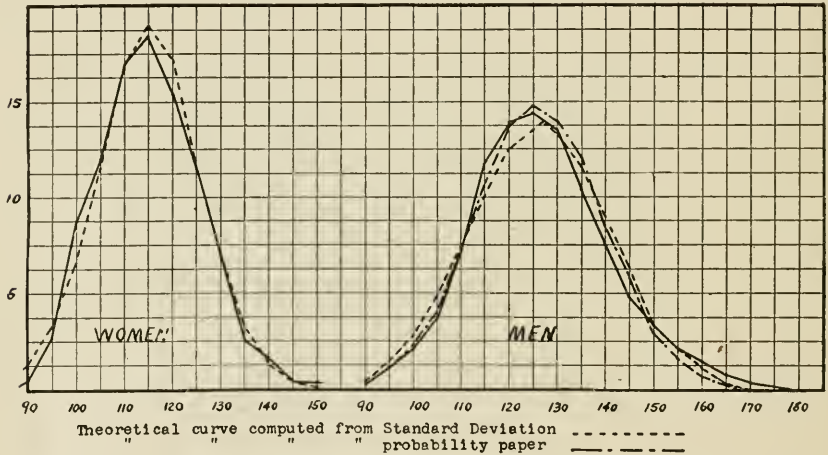


Fig. 2.—The solid lines represent smoothed frequency curves for 5,807 women and 2,930 men students from 16 to 40 years of age. The dotted lines represent the corresponding theoretical curves computed mathematically. The dash and dot lines represent theoretical curves obtained by plotting the straight lines in Figure 3. The ordinates are percentages and the abscissae, pressures.

and of the women's curve for 16, where modes fall on 105, 115, and 135 mm. Particularly in the case of the women, where the readings were made with great care, it is hard to understand why the sides of the graph should show so little evidence of deformity when the top is deeply notched. If we could be sure of the figures, the bimodality of the curves might lend support to my theory that there are perhaps two types or varieties of the human species: one in which hypertension will not develop, except perhaps in old age; and the other in which it, or allied disturbances, develop early on the basis of a congenital predisposition. Unfortunately for this argument, even when there

are two groups, if their modes are close together the fused curve will be unimodal. This is shown in Figure 7, where the solid line represents the distribution curve for all the women and men, taken together. Here we see how two groups 'with different modes can fuse so that

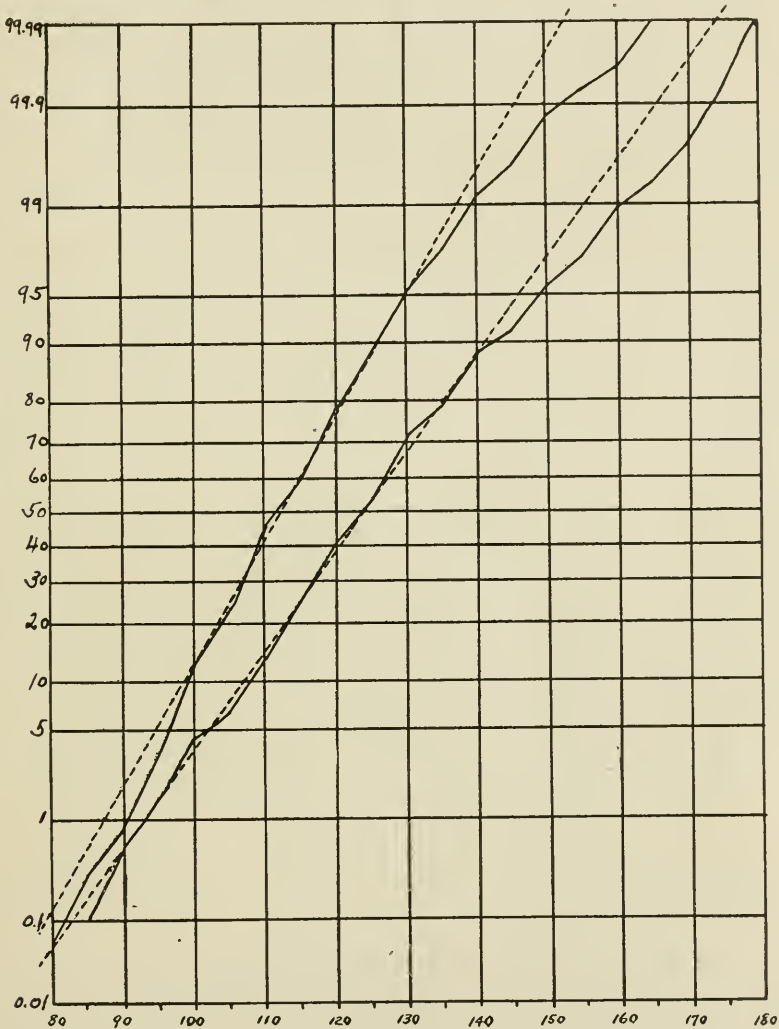


Fig. 3.—Shows the data for men and women students plotted on probability paper. The ordinates represent percentages; the abscissae, pressures. If the observations followed the law of probability exactly they would plot as a straight line.

the resultant curve is similar in form to the individual ones. If there had been equal numbers of men and women in the two groups the combined curve would have been like that in the corner of Figure 7.

That curve, with its mode at 120, probably comes pretty close to representing the distribution of blood pressures in Americans between 16 and 36.

DISCUSSION

Before entering on a discussion of the findings it might be well first to answer an objection which has been made by many of the friends who have seen these data during the last year, and which may be made again. These men felt that the higher figures should be disregarded because it is well known that the pressure in some people varies from hour to hour, and that it often goes up under excitement.¹³

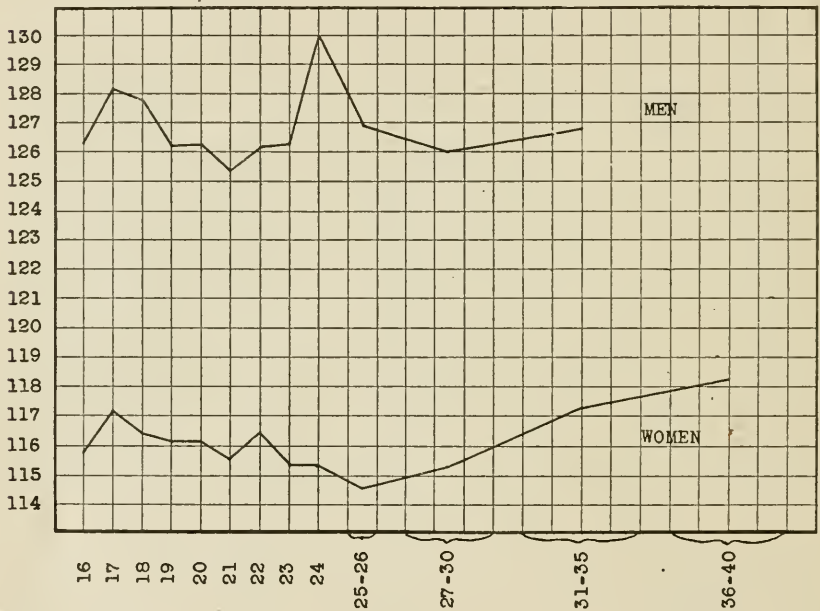


Fig. 4.—Shows the curves of average pressure for men and women between the ages of 16 and 40.

While this is true, it must be remembered that all the students were examined in the same way so that all were equally subject to excitement; and yet one developed a pressure of 115 mm., another 155, and another a fainting spell with a pressure of 80. By examining the men with 155 and 80, day after day, we might, perhaps, get figures more nearly in accord with our ideas of what they should be, but such juggling of disturbing data is not permissible in a scientific study. If we are going to measure some over again we must measure them

13. O'Hare: *Am. J. M. Sc.* **159**:369, 1920.

all. On another day some of the high pressures would undoubtedly be lower, but some of the borderline ones and some even of the high ones would be found higher.

Unfortunately, I have not been able to measure these students over again, but I have taken a series of carefully recorded measure-

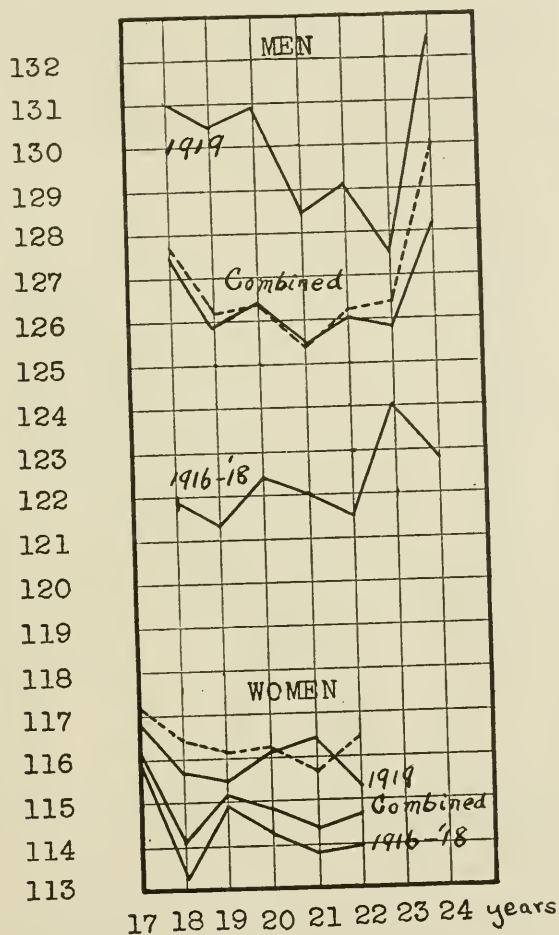


Fig. 5.—Showing differences in the averages in the different age groups, for 1919 and for 1916-1918. The ordinates are pressures and the abscissae, ages. The middle lines show averages for the combined data of 1916-1919. These averages were obtained after grouping the data by stages of 5 mm. The dotted lines are exact averages taken from Figure 4.

ment on 100 consecutive office patients, 50 women and 50 men, who were observed twice at varying intervals of time. In spite of the fact that many of these people took treatment: rest cures, etc., in the interims between the two measurements; and in spite of wide individual

variations, there was a difference of only 0.07 mm. between the first average and the second. On the second examination, the women averaged 1.94 lower, while the men averaged 2.08 mm. higher. These findings make me feel that if I could measure the students over and over again with great care, individual variations would probably balance each other so that the frequency polygons and averages would remain about the same. Furthermore, it must be remembered that not only can the excitement account for an increase in pressure but a tendency to hypertension will often account for the excitement. The phlegmatic individual with a normal pressure is perhaps bored by the proceeding, while the keen hypochondriac with an over sensitive

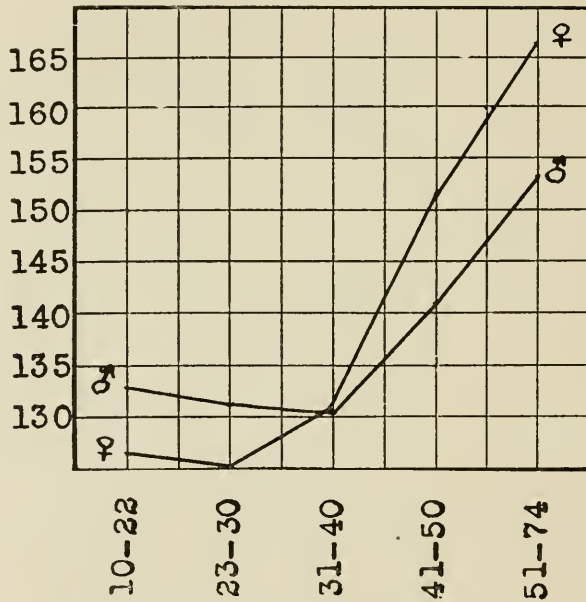


Fig. 6.—Shows yearly averages on 1,000 office patients. The abscissae represent age groups. The curve for the women is below that for the men before 40, and above it afterwards.

and erratic vasomotor system is afraid that serious organic lesions will be found; he gets nervous and his pressure goes up. It is not improbable also that it is the man with the hypertensive diathesis who is most likely to show the wide variations. One of the characteristic things about these people is their vasomotor instability; they blush, they blanch, they feel hot in one part of the body and cold in another. There is good reason, therefore, for expecting their blood pressures to be more variable than those of the people with good vasomotor control. Furthermore, we probably get a much better idea about a man's vasomotors and his blood pressure when he is under a little strain than

when he is under the most favorable conditions, just as we can judge better about a man's heart after exercise; about his pancreas after feeding sugar, and about his kidneys after feeding salt or urea. At any rate, the essential point is that if a thousand young people are examined on one day under the same conditions, with the same instruments and the same examiners, some will be found with low pressures, many with medium ones and a considerable number with high ones.

The next thing to do before we can discuss the incidence of hypertension in these students is to define the limits of normal. Such a

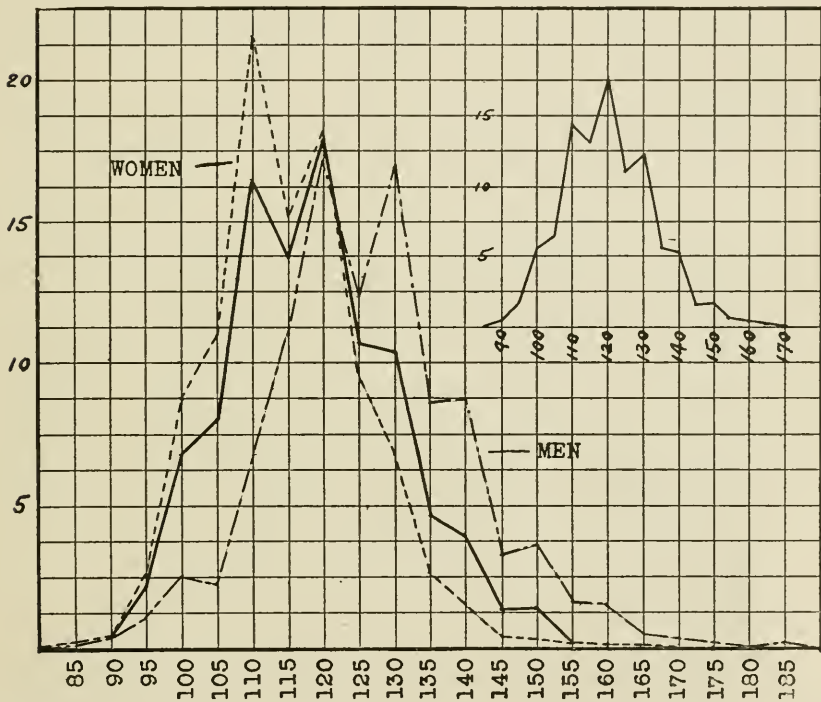


Fig. 7.—To show that a unimodal curve can conceal the fact that there are two groups present with modes at 115 and 125 mm. The solid line represents the distribution for 8,737 men and women together. The small curve in the corner shows this distribution as it would have been if there had been equal numbers of men and women.

definition will be of interest not only to the student but to the clinician and to the life insurance medical director. There are several ways in which we can approach this problem. We can find the average and take certain limits on each side of it; we can study the points of divergence of the actual from the theoretical frequency curve, and best of all, we can watch a large series of borderline cases to see which

ones have, or later develop, other signs of cardiovascular-renal peculiarity.

The arithmetical mean for the men is 126.5; for the women 115.0 mm. If we exclude the data below 100 and above 130 we get 120.3 for the men and 114.4 for the women. Figure 3 shows that the median or mid number of the men is 123.5; for the women it is 112.5. The median is often a good measure of central tendency because it is not so much affected by the widely divergent figures as is the mean. Mathematicians often estimate the "probable error" or limits within which the middle 50 per cent. of the data will lie. For the men these limits are 115-132.5 for the median and 116.5-136.5 for the mean. For the women the limits are 105-119 for the median and 107.8-122 for the mean. Judging by clinical experience, these figures do not seem to be very helpful. They suggest that the measures of central tendency are placed too high for the men. They show again very clearly the great difference in the dispersion of the data for men and women.

If we turn to Figure 3 we note that the data for the women follow the law of probability between limits of 100 and 130 mm.; the data for the men follow the law between 90 and 140 mm. That suggests strongly that between these limits variations from the average are due to many small errors attendant on the method of measurement, the condition of the subject, etc. Above and below these limits the divergence of the found from the expected would appear to be due to pathologic causes. This is shown still more clearly in the curves from office patients. See figure 8. In these, there can be no doubt but that the humps off to the right, in some instances entirely separate from the main polygon, represent pathologic cases.

Let us turn now to the limits which have been set by various writers in the past. One often hears it stated that the pressure for any age is roughly 100 plus the age. Figure 4 shows at a glance that that rule cannot be used in youth when the pressure is declining with age. The life insurance statistics show that it cannot be used even for older men. According to MacKenzie, the normal range for men between 15 and 39 is 26 mm. Taking our mean of 126.5, the limits would be 113.5 and 139.5. Fisher thinks 12 mm. above the average for the age is a permissible deviation. For the women this would make 127 the upper limit. Janeway¹⁴ was inclined at first to take 150 or 160 mm. as the upper limit of normal, but in 1915¹⁵ he said, "I am inclined to revise my former opinion and to agree with Cook and Lauder Brunton before him that over 135 mm. up to middle life,

14. Janeway: Arch. Int. Med. **12**:755 (Dec.) 1913.

15. Janeway: Bull. Johns Hopkins Hosp. **26**:341, 1915.

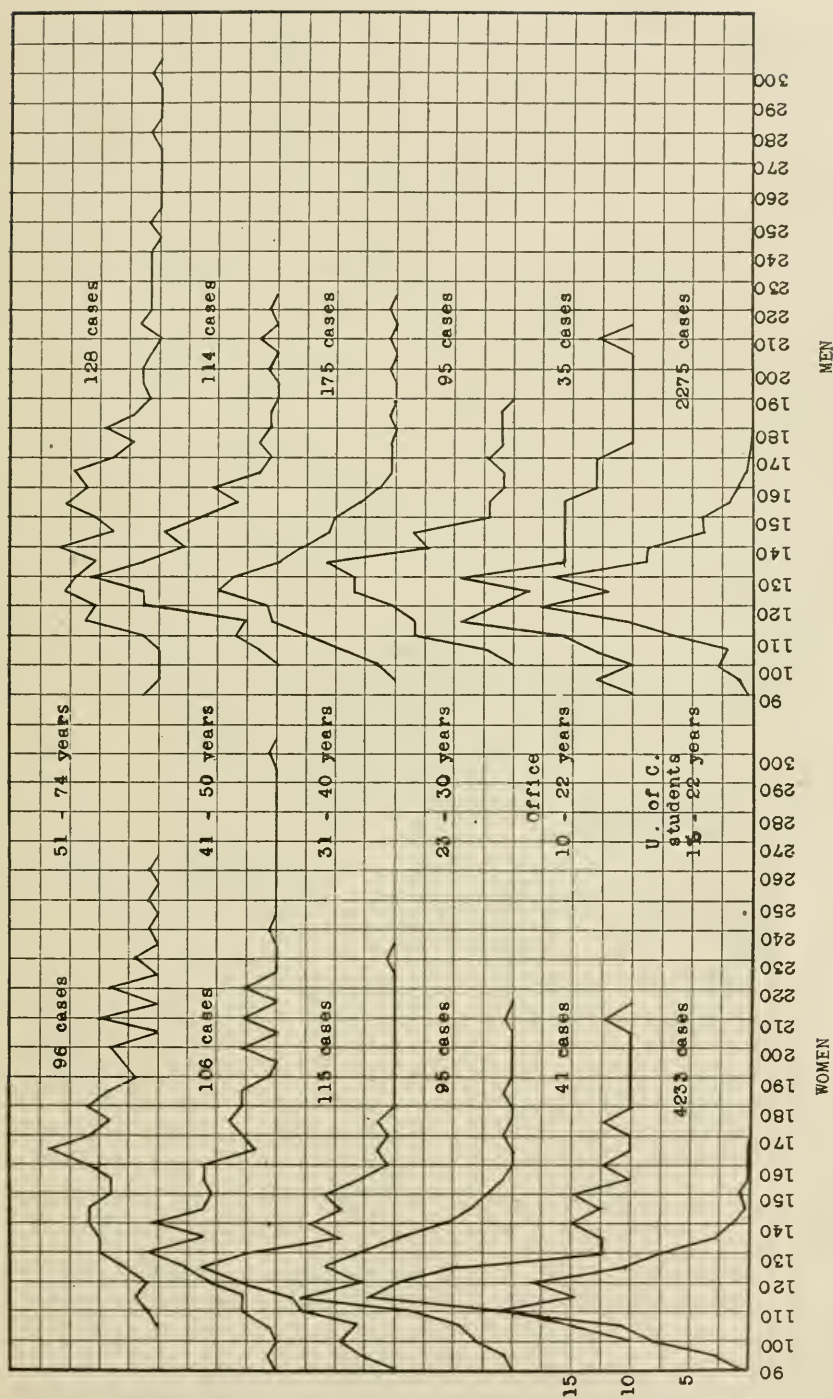


Fig. 8.—To show frequency distribution of blood pressures in 1,000 office patients at different ages, compared with the distribution of 6,508 students between 16 and 22. The changes in the women's curve after 40 is well shown.

and 145 to 150 mm. thereafter should be regarded as pathologic hypertension if found on repeated examinations." In a series of 7,872 office patients, he found 11.1 per cent. with pressures of 165 mm. or over. Lee,⁸ who studied Harvard freshmen, places the upper limit at 140 mm. In his series of cases he had 12.8 per cent. exceeding that figure; at the University of California there were 20.4 per cent.

It seems pretty clear from a study of the literature that with the passage of time and the accumulation of knowledge about the subject, the tendency is to lower the upper limit of normal. A few years ago I would have accepted 135 or 140 mm. as the upper limit of normal for young people, but I have seen so many men and women in whom pressures between 130 and 135 were associated with irritable, enlarged hearts, anginoid pain, dizziness, morning headache, cyanotic hands, transient albuminuria and other typical findings, that we feel that the upper limit for the men should be placed at 130. Theoretically the limit for the women should be 5 or 10 points lower, but practically it would seem to be a little higher, perhaps about 127. This may be because women often appear to tolerate hypertension better than the men do.

TABLE 3.—PERCENTAGES INCLUDING AND OVER THE DIFFERENT PRESSURES IN THE CALIFORNIA STUDENTS; IN THE STUDENTS STUDIED BY BARACH AND MARKS, AND BY LEE (SEE BIBLIOGRAPHY); IN ONE THOUSAND OFFICE PATIENTS, AND THIRTEEN THOUSAND SEVEN HUNDRED AND EIGHTY-NINE MIDDLE AGED MEN APPLYING FOR INSURANCE

Blood Pressure, Mm.	Univ. of California Students			Barach and Marks, 18-31 Years	Lee	Office Patients, 10-40 Years		13,789 Insurance Applicants, Men
	Men		Women			Men	Women	
	1916-1919	1916-1918						
130	46.0	36.0	12.0	47.0	53.0	38.0	9.0
135	28.9	5.1					
140	20.4	12.0	2.4	24.0	12.8	26.0	21.0	
145	11.5	0.8					
150	5.6	4.7	0.4	9.4	14.0	12.0	

According to Table 3, the 130 mm. limit seems reasonable enough so far as the women students are concerned; but it seems as if it must be too low for the men. It may be, of course, that the sampling was bad, and that in later years we will get figures even lower than those obtained in the years before 1919. It is interesting to see how much more frequently hypertension is found in women who go to a doctor's office than in women who go to college. The difference is not so marked for the men. At first sight these figures may seem incredible, but let us check them in several ways. At the office I have found that practically all of the men with pressures over 130 show other signs of cardio-vascular peculiarity. The insurance exam-

iners say that 83 per cent. of the applicants with pressures over 150 can be rejected for albuminuria, cardiac hypertrophy, etc. (1917, p. 252). Table 2 in my article on drafted men shows how rapidly the percentage of such abnormalities rises with the increase of blood pressure: from 54 per cent. at 130 mm. to 100 per cent. at 166 mm. Lee found other cardiovascular troubles in twenty-two out of the eighty-five students with pressures over 140 mm. In this group of 662, 5.1 per cent. had albuminuria. MacLean¹⁶ who studied 60,000 soldiers (already selected) found albuminuria in 12.6 per cent. after exercise. Unfortunately, the statistics of the provost marshal general on the draft do not help us much because so many of the men did not have a complete examination, but were dismissed as soon as some obvious disqualification such as an amputation, a stiff joint or a large hernia became apparent. Moreover, the work was done hurriedly; blood pressures were rarely taken and analyses of the urine were rarely made. In 1917, 2,521 of those passed into the army had almost immediately to be treated for valvular heart disease.¹⁷ Even among the carefully picked aviators, Whitney¹⁸ found 5 per cent. who had to be rejected on this score. Hence it is that the 13.1 per cent. rejected for heart disease by the local boards and induction examiners¹⁹ did not include all even of the obvious and striking cases of cardiac disease, let alone those with nephritis and hypertension. In some states, where the examination was probably conducted more carefully, the figure rose as high as 22.8 per cent. Under the circumstances, then, remembering how highly diluted these percentages are by the number of cases in which the heart was not examined, we must be impressed by the high incidence of cardio-vascular disease in the young men of the nation.

In the discussion on Dr. Fisher's paper, Dr. Van Wagenen pointed out that 40 per cent. of 17,500 deaths studied by his company were traceable to cardiovascular-renal disease in one form or another, (1917, p. 259). As he said, "It makes little difference to us whether a man begins the circle of disease with an apoplexy, a chronic heart trouble or kidney disease; if he lives long enough he will develop all those different troubles." As we know that many of those who come to their end on account of hypertension, arteriosclerosis, heart and kidney disease are reported as having died of pneumonia, acute dilatation of the stomach, etc., we must suspect that at least one-half of all deaths are attributable to chronic cardiovascular disease. If, as I believe, the

16. Maclean: *Brit. M. J.* **1**:94, 1919.

17. Second Report of the Provost Marshall General, Washington, 1919, p. 424.

18. Whitney: *J. A. M. A.* **71**:1389 (Oct. 26) 1918.

19. War Department Bull. 11, 1919, p. 84. Physical Exam. of the First Million Draft Recruits.

tendency towards these troubles often exists from birth, then, if we look carefully enough, we need not be surprised if we find slight hypertension or other related peculiarities in about 50 per cent. of young men. I shall discuss shortly a possible explanation for the fact that these defects show up so much later in the women.

The main hope in attempting to define an upper limit of normal is that we may be able later to say from experience that if a boy has a pressure above a certain figure he is going, if he lives long enough, to develop definite cardio-vascular disease. It may prove impossible to prophesy about those with low pressures because some, perhaps, will develop hypertension in middle life, while others will live on past 70 with remarkably good arteries and kidneys. We will have to follow for the next forty years a large series of young men with apparently normal cardiovascular systems before we can say how many develop the degenerative diseases late out of a clear sky, and how many appear to develop them late, but really have shown a few signs and symptoms from childhood. The insurance directors who re-examine and follow up their cases will undoubtedly be able later to answer many of these questions. In the meantime we can learn a great deal by taking very careful histories on those past middle age who come now with hypertension. Not infrequently I get histories of "heart trouble," "kidney trouble," polyuria, dyspnea, palpitation, anginoid pain and "nervous breakdowns" dating back into childhood or years before the patients discovered their hypertension. Naturally in many instances this history can be gotten only from the mother. She will recall diagnoses and symptoms which caused her great anxiety during trying periods of her child's development. A review of the literature shows that chronic nephritis is not so uncommon in childhood and in many of the reported cases no infectious or other cause could be found.²⁰ We must analyze a large series of these histories to see whether the children of people with cardio-vascular disease are more likely to develop endocarditis and nephritic infections after the exanthemata than are the children of those with good hearts and kidneys. The bacteriologists are now awaking to the fact that the virulence of organisms is not the only variable and the resistance of the guinea-pig a constant. They are beginning to suspect that particularly when studying susceptibility to chronic infections, they must have to develop pedigreed strains of animals whose reactions to disease will be uniformly high or uniformly low. It is unfortunate that his training makes the city specialist look at his patient as a lone man making a short "to a finish" fight with bacteria. In many instances what the physician sees is but one round

20. Hill: *Am. J. Dis. Child.* **14**:267 (Sept.) 1917.

Berkeley and Lee: *Am. J. Dis. Child.* **13**:354 (April) 1917.

Judson and Nicholson: *Am. J. Dis. Child.* **8**:257 (Sept.) 1914.

of a fight which began years before and which will drag on until old age appears on the scene. Moreover, the patient is not alone but is surrounded by the shades of many ancestors who are making the fight hopeless either for him or for the bacteria.

SEXUAL DIFFERENCE

We come now to a discussion of the big sexual difference in blood pressure. As I stated above, the distribution curves suggest strongly that if there are two types in the community, we may say that the women before the menopause appear to be composed almost entirely of the type endowed with a low pressure. This uniformity is seen also in the fact that half of their readings fall within limits of 7 mm. on each side of the average. The interesting thing is that after 35, the women's distribution curve widens out so that it comes to resemble that for the men. We know that this big increase in blood pressure often comes at the menopause when the ovaries atrophy. It suggests that in some way the ovary is able to cover up or hold latent the tendency to hypertension which we will presume the women inherit equally with the men. Another possibility is that hypertension is a defect linked up, as are some other well known defects, with sex chromosome. This might explain why it appears early in the males who have only one such chromosome. The females have two, one of which may be normal and able to neutralize the defect in the other. With the atrophy of the ovary at the menopause the defect may appear, much as the plumage of a cock appears sometimes in hens with diseased ovaries. Much in favor of these theories is the observation that hypertension often develops early in women who show signs of insufficient ovarian function, such as scanty and painful menstruation, sexual anesthesia, male distribution of body hair, infantile uterus, etc. We shall have to check these observations statistically to see whether or not the correlation is close enough to be significant. Careful studies will have to be made also on families to ascertain the incidence of hypertension (1) when neither parent has cardiovascular disease; (2) when one has it, and (3) when both have it. I have already collected some records which show that the incidence of hypertension in children is likely to be very high when both parents show the defect.

We must also study in childhood the beginning of the sexual difference in blood pressure. Unfortunately, although considerable work has been done on blood pressure studies in children, I can find no data so arranged that we can say when the divergence appears. Some writers give their impression that it appears about the age of puberty, which is just what we should expect. If this point is settled definitely we shall be able to associate hypertension very closely with the evolution of sex and thus with the glands of internal secretion. It is inter-

esting to note in Figure 8 that the big increase in the incidence of hypertension comes 10 years later in the men than in the women. Apparently, the strenuous life has less to do with this disease than has the quieting down of the sexual functions.

PROGNOSIS

What does slight hypertension mean in youth? Many will answer absolutely nothing. We know that these people can live out their three-score and ten and can work hard physically and mentally. I agree entirely with Sir James MacKenzie, Sutherland²¹ and others who decry the tendency on the part of many physicians to treat these young people with "functional" cardio-vascular disease energetically with drugs, restricted diets and restriction of activities. Until we know more about the causation of the trouble, it does not seem to me that we have the right to prescribe minutely or to threaten the patient with disaster if he does not follow our instructions. Many of these boys enjoy athletics and we have as yet no data to show that such exercise will shorten life. We have no data showing that a protein poor diet persisted in for forty years will lengthen life. About all we can say is that as these people often tend to break down nervously, they should live within their means of strength; they should get enough sleep and rest, and should avoid nervous strain.

Although the experience of men like MacKenzie who have watched young people with murmurs, tachycardias, extrasystoles, transient albuminurias, etc., for long periods of time shows that the prognosis is generally good as regards the immediate future, I cannot accept their statements that these things mean nothing at all. In one instance a patient expressed it very well. He was a well built athletic young man of 24, a nervous wreck on account of a slight hypertension associated with extrasystoles and palpitation. He said, "All doctors who see me tell me to forget it, that there is nothing the matter with me; but I know that my uncles all had this sort of thing and they all died young with myocarditis. Now, if I live long enough won't I go the same way?" The fact that some obscure infection took him off three months later suggests that his presentiments of impending dissolution were not so unfounded and neurotic as most of his physicians thought they were.

Although people with hypertension can round out fairly long lives, the statistics of the life insurance companies show that in the aggregate this disease definitely shortens the expectancy of life. This is shown convincingly in Table 4 made up from data embodied in Fisher's paper (1915). This shortening of life is the more striking when we remember that these deaths took place within a period of at most eight years following the application for insurance. It is also signi-

ficant that it was cardiovascular disease which took off at least 78 per cent. of the men with the high pressures.

TABLE 4.—LIFE EXPECTATION IN PERSONS WITH HYPERTENSION *

	Per Cent.
Percentage of expected deaths according to American Actuarial tables.....	100
Pressures of 105 and under.....	47
Pressures of 106 - 110.....	65
Usual experience in accepted cases.....	86
10-14 mm. over average for age.....	114
15-24 mm. over average for age.....	181
25-34 mm. over average for age.....	205
35-44 mm. over average for age.....	246
45-59 mm. over average for age.....	254
60 plus mm. over average for age.....	450

* 2,857 cases were observed for periods of time ranging up to eight years.

Perhaps, we may look at it in this way: half of us are to depart from this world on account of cardiovascular disease. An ignorant, insensitive laborer may discover it at 60 when he has a stroke, or an acute decompensation of the heart. A business man, careful of his health, has his handicap discovered about the age of 40. A still more careful man may, perhaps, recognize in the circulatory disturbances of his little children the fore-runner of the process which, later in life, is to cause trouble. Many will ask, what good can come out of such pessimistic views? Several advantages may accrue. In the first place, we may learn a great deal about this disease by following it, not for a few months, but for a life-time or through several generations. Secondly, if we ever find specific methods for combating the process, it will probably be helpful to begin early; and thirdly, if we find that these diseases run through a lifetime we may spare our patients endless annoyance and financial loss in attempts at cures through tooth pulling, tonsil removing, purgation, sweating, use of drugs, high frequency currents, etc. Much light might be thrown on the etiology of the disease by studying the nature of the inheritance. Thus, if it be shown that not infrequently a man with a "pure hypertension" can have a father with marked arteriosclerosis, a mother with myocarditis, an uncle with nephritis and children with orthostatic albuminuria, and "functional murmurs," we will be in a position to say that these manifestations are probably all related.

It is to be desired that in the future those who write on this subject will give not only a few averages, but tables of frequency distribution so that their data can be used again and studied in other ways by subsequent observers.

SUMMARY

A statistical analysis has been made of the blood pressures in 8,737 University of California freshmen and 1,000 office patients.

The mathematical treatment of these data suggests that pressures over 130 mm. for the women and over 140 mm. for the men are abnormal. The arithmetical mean for women between 16 and 40 was 115 mm.; for men, 126.5 mm.

The blood pressure in young women is much more uniform than in men. The range for the women was practically from 85 to 155; for the men it was from 90 to 175. Fifty per cent. of the women's readings fell between 105 and 119 mm.; 50 per cent. of the men's fell between 116.5 and 136.5 mm.

High blood pressure appears earlier and to a greater degree in young men than in young women.

The average blood pressure in the women rose from 16 to 17; then dropped to 25 and after that rose rapidly. Little can be said about the men's yearly averages on account of disturbances in sampling brought out by the world war. Averages from the office patients showed that the pressure for women drops from puberty to 25, after which it rises so rapidly that the women catch up with and pass the men after 40. Apparently changes in the gonads of men and women have more effect on the blood pressures than has the strenuous life.

It is suggested that hypertension is based on a hereditary peculiarity. Its manifestations appear to be suppressed in women as long as the ovaries function well.

From clinical experience it would seem that pressures over 127 in women, and over 130 in young men, are indicative of a hypertensive diathesis which is associated with many typical symptoms and findings.

Fifty out of a hundred men will die of cardiovascular disease. This makes its appearance at different ages in the different men. The writer believes that careful examination would show the beginnings of such disease in childhood and youth, even in those individuals who are to round out a fairly long life. He believes that a hereditary predisposition is the most important etiologic factor.

Suggestions are made for further work along the lines of these hypotheses.

I wish to thank Dr. Robert Legge and Dr. Ruby Cunningham for their cooperation and their kindness in giving me access to their excellent records.

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CHICAGO

FURTHER STUDIES ON THE ELIMINATION OF TAURINE ADMINISTERED TO MAN.*

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(Received for publication, March 3, 1920.)

The question as to the fate of certain amino-acids when given in large doses has been the subject of numerous papers. The evidence that the portion which is excreted by the kidney is combined with urea in the form of uramino-acid rests on the basis that this compound has been isolated from urine.

Schultzen¹ isolated sarcosincarbamic acid from the urine of a dog fed with sarcosin, but the quantitative differences in yield noted by Baumann and von Mering,² Salkowski,³ and Schiffer⁴ on repeating this experiment can hardly be attributed to individual variability of the experimental animals. The ease with which amino-acids combine with urea to form the corresponding uramino-acid⁵ would lead one to suspect that the latter when found in urine was not preformed in the body but a resultant of the process used for its isolation, and the quantity obtained dependent upon the particular technique used. Dakin⁶ fed inactive tyrosine and phenylalanine to cats and although he found, in accordance with results previously obtained by Blendermann,⁷ uramino-acids in the urines of his experimental animals he recognizes the possibility that these substances were formed in the process of isolation from the unchanged amino-acid present in the urine. When the urine was acidified with acetic acid as soon as passed the yield of uramino-acid was so small as to be insufficient for identifica-

* Aided in part by a grant from the George Williams Hooper Foundation for Medical Research.

¹ Schultzen, O., *Ber. chem. Ges.*, 1872, v, 578.

² Baumann, E., and von Mering, J., *Ber. chem. Ges.*, 1875, viii, 584.

³ Salkowski, E., *Z. physiol. Chem.*, 1880, iv, 100.

⁴ Schiffer, J., *Z. physiol. Chem.*, 1881, v, 257.

⁵ Weiland, W., *Biochem. Z.*, 1912, xxxviii, 385. Lippich, F., *Ber. chem. Ges.*, 1908, xli, 2974.

⁶ Dakin, H. D., *J. Biol. Chem.*, 1910, viii, 25, 35.

⁷ Blendermann, H., *Z. physiol. Chem.*, 1882, vi, 234.

tion. The failure of Rohde⁸ to obtain by analytical methods, after decomposing the urea with urease, evidence of the presence of uramino-acid in the urine of a cat injected with phenylalanine, indicates that the α -ureidiod- β -phenylpropionic acid isolated by Dakin⁹ in a similar experiment was formed after the urine had been passed.

The experiments of Salkowski¹⁰ on the elimination of taurine have been of particular interest to us. He found that taurine when taken by mouth was eliminated in large part in the urine. This was determined by estimating the increase in neutral sulfur. The substance which he isolated was not taurine but taurocarbamie acid. Experiments reported by Schmidt, von Adelung, and Watson¹¹ confirmed Salkowski's findings with regard to the increase of neutral sulfur. It, however, appears to us in view of the doubt which exists as to the formation of uramino-acids in the body that the taurocarbamie acid isolated by Salkowski was formed after the urine had been passed. Our data presented below show that taurine is excreted in the free state and not combined with urea as taurocarbamie acid.

Since taurine yields its nitrogen quantitatively in 4 minutes¹² when shaken with nitrous acid while the uramino-acid gives off its nitrogen but slowly, use was made of the method of Van Slyke¹³ for the determination of α -amino nitrogen and the nitrogen due to slowly reacting amines in the urine of a subject to whom taurine was administered. If taurine is excreted in the urine in the free state an increase in α -amino nitrogen over the normal output should be noted, but if it is present as taurocarbamie acid the nitrogen obtained from the slowly reacting amines should show a corresponding increase. Preliminary experiments in which taurine and taurocarbamie acid were added to urine showed that taurocarbamie acid was not split by urease,¹⁴ and that the added taurine could be quantitatively estimated from the increase of α -amino nitrogen.

A subject was placed on a constant diet and the normal excretion of total nitrogen, amino-acid nitrogen, and sulfur determined.

⁸ Rohde, A., *J. Biol. Chem.*, 1918, xxxvi, 467.

⁹ Dakin, H. D., *J. Biol. Chem.*, 1909, vi, 235.

¹⁰ Salkowski, E., *Virchows Arch. path. Anat.*, 1873, lviii, 460, 580; *Ber. chem. Ges.*, 1872, v, 637.

¹¹ Schmidt, C. L. A., von Adelung, E., and Watson, T., *J. Biol. Chem.*, 1918, xxxiii, 501.

¹² Foster, M. G., and Hooper, C. W., *J. Biol. Chem.*, 1919, xxxviii, 355.

¹³ Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 125.

¹⁴ Previously noted by Mateer, J. G., and Marshall E. K., Jr., *J. Biol. Chem.*, 1916, xxv, 297.

TABLE I.
Subject I.

Day of experiment.	Total nitrogen.	α -amino nitrogen.	Nitrogen from slowly reacting amines.*	Total sulfur.	Total sulfates.	Neutral sulfur.	Remarks.
	gm.	gm.	gm.	gm.	gm.	gm.	
1	10.1	0.38	0.11	0.80	0.66	0.14	10 gm. taurine by mouth. S = 2.56 gm.
2	10.3	0.44	0.05	0.82	0.67	0.15	
3	9.3	0.38	0.10	0.72	0.58	0.14	
4	11.0	1.12	0.12	2.49	0.70	1.79	
5	9.8	0.46	0.09	0.84	0.63	0.21	
6	9.3	0.38	0.09	0.78	0.56	0.22	

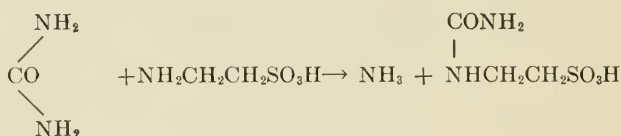
* Calculated on the basis of 4 minutes.

TABLE II.
Subject II.

Day of experiment.	Total nitrogen.	Urea nitrogen.	Urea nitrogen in total nitrogen.	Ammonia nitrogen.	Ammonia nitrogen in total nitrogen.	Remarks.
Experiment A.						
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>gm.</i>	<i>per cent</i>	
1	9.0	7.4	82	0.58	6.4	3.3 gm. taurine by mouth in 10 (hourly) doses.
2	9.4	7.4	79	0.54	5.7	
3	8.7	6.7	77	0.66	7.6	
4	9.4	7.3	78	0.57	6.1	14 gm. taurine by mouth in 10 (hourly) doses.
5	10.4	7.3	70	0.79	7.6	
6	9.3	7.4	80	0.65	7.0	
Experiment B.						
1	6.6	5.7	86	0.44	6.7	15 gm. taurine intravenously.
2	7.5	6.2	83	0.52	6.9	
3	8.7	7.0	80	0.51	5.9	
4	9.5	7.0	74	0.48	5.1	
5	8.2	7.2	88	0.43	5.2	15 gm. taurine intravenously.
6	8.8	6.8	77	0.46	5.2	
7	8.1	6.4	79	0.56	6.9	

He was then given 10 gm. of taurine by mouth and similar determinations were carried out for this day as well as for several succeeding days. The results are given in Table I. It is at once evident that ingestion of taurine has resulted in an increase of both neutral sulfur and α -amino nitrogen, in amounts which correspond to about 63 per cent of the ingested taurine. There is no increase in the slowly reacting amines, indicating that no appreciable amount of taurine has combined with urea to form taurocarbamic acid. On the basis of increase in total nitrogen excreted, the amount of taurine eliminated is greater than that calculated from the sulfur or α -amino nitrogen. Since there is a greater variability in the total nitrogen figures the latter method of calculation seems the more accurate.

Several other experiments were carried out to determine the effect, if any, of the ingestion of taurine on the excretion of urea and ammonia in urine (Table II). If urea and taurine combined *in vivo* and the reaction follows the same course as *in vitro*



a decrease in urea and a corresponding increase in ammonia nitrogen should be expected. Within the normal variability the amounts of urea and ammonia excreted are not affected by the administration of taurine. These figures support those of the previous experiment and point to the conclusion that taurocarbamic acid is not a metabolic end-product when taurine is ingested.

SUMMARY.

1. Determinations of neutral sulfur and amino-acid nitrogen in the urine of an individual given 10 gm. of taurine by mouth show a close agreement in the increase of α -amino nitrogen and neutral sulfur over the control period, indicating that taurine is not eliminated as taurocarbamic acid.

2. Administration of taurine in large doses does not result in any marked changes in the excretion of urea and ammonia.

ON THE PROTECTION AFFORDED TO RED CELLS AGAINST HEMOLYSIS BY EOSIN *

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In 1900 Raab¹ and von Tappeiner² found that if infusoria are placed in a very dilute solution of a fluorescent dye (acridin) and kept in the dark, the organisms are not injured, but on exposing them to diffuse sunlight, death results. The researches of von Tappeiner and Jodlbauer,³ and others have established that the photodynamic effect, associated with fluorescent dyes, is toxic not only for unicellular organisms, but also for enzymes, bacterial toxins, immune bodies, blood cells and even for the higher animals. The effect obtained by placing the photosensitive organisms in the dye and keeping them in the dark for some time is no greater than when the dye is added just before the organisms are exposed to sunlight, and it is immaterial, for the purpose of laking red cells, whether the stain is within the cell or not. Fluorescence is necessary for photobiologic action but the quantitative effect is not proportional to the amount of fluorescence. It is necessary that the fluorescent solution be in intimate contact with the substance on which it is to act; absorption of fluorescent waves alone does not suffice to produce photodynamic effects. The rôle played by oxygen in this phenomenon is still a matter of dispute, but the evidence seems to indicate that its presence is necessary.

Fluorescence is not limited to certain dyestuffs but is a property of many compounds found both in animal and vegetable life and these share with the dyes the common property of sensitizing protoplasm for photodynamic action.

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¹ Ztschr. f. Biol., 1900, 39, p. 524; 1903, 44, p. 16.

² München. med. Wchnschr., 1900, 47, p. 5.

³ Ibid., 1904, 51, p. 737; Deut. Arch. klin. Med., 1904, 80, p. 427; 1905, 85, p. 386; 1906, 86, p. 468 and p. 479; Kudo and Jodlbauer: Biochem. Ztschr., 1908, 13, p. 24; Harzbecker and Jodlbauer: *ibid.*, 12, p. 306; Neuberg: *ibid.*, 13, p. 305; von Tappeiner: *Ergeb. der Physiol.*, 1909, 8, p. 698; Neuberg and Galambos: *Biochem. Ztschr.*, 1914, 61, p. 315.

Busck⁴ and later Sellards⁵ found that addition of certain substances, such as blood serum and egg-white, to solutions of photobiologic sensitizers results in decreasing or completely inhibiting toxic action, but no protection was afforded by the addition of glucose, starch or gelatin. This interesting observation appeared to us to demand further consideration, since there are definite chemical differences between the inhibiting and noninhibiting substances. The inhibitory effect cannot be attributed to the amphoteric character or the colloidal nature of the protective substances. There is a striking difference in the nature and content of the amino acids of gelatin and egg albumin. The former wholly lacks tyrosin, tryptophan and cystin, and contains only minimal amounts of alanin, serin, aspartic acid, phenylalanin and histidin.⁶

Former work⁷ on the protection afforded to immune bodies against the toxic action of ultraviolet light showed that the aromatic amino acids have the power of absorbing these waves, and since action by light cannot take place without absorption, the presence of these amino acids in the protein molecule is essential for this reaction. In the present work we have sought to find a correlation between the protection afforded to red cells by certain proteins against lysis by eosin and the nature of the amino acids in the molecule and our results indicate that the presence of tyrosin or tryptophan determines ability to protect.

The experiments were carried out with eosin (Grübler's w. g.) and red blood cells were used to indicate toxic action; 0.5 c.c. of a 5% saline suspension of red cells (sheep or ox) was placed in each of a number of small test tubes and to each, 1 c.c. of a 1:10,000 eosin in salt solution was added. The substances to be tested for protective action were likewise made up in salt solution in the concentrations as given in the table and the reaction adjusted to P_H 7.5-8.0. The tubes containing the suspension of red cells, eosin and substance to be tested for inhibitory action and also a number of control tubes were placed in the sunlight for a period of 30 minutes and after exposure immediately placed in the ice chest. The tubes were inspected at the

⁴ Biochem. Ztschr., 1906, 1, p. 425.

⁵ J. Med. Research, 1918, 38, p. 293.

⁶ Fischer, Levene and Aders: Ztschr. physiol. Chem., 1902, 35, p. 70; Levene and Beatty: Ztschr. physiol. Chem., 1906, 49, p. 252.

⁷ Harris and Hoyt: Science, 1917, 46, p. 318; University of Cal. Pub. Path., 1919, 2, p. 245; Hill and Schmidt: J. Infect. Dis., 1919, 25, p. 335.

end of 3 hours and again after 18 hours to determine the amount of lysis. Only in those tubes which after 3 hours showed that some hemolysis had taken place, was a slight increase noted after 18 hours. A certain number of tubes similar to the above kept in the dark in order to rule out the possibility of any unlooked for factor being concerned in the reaction.

From the table it will be noted that protein substances, other than egg-white and blood serum, namely, ovomucoid, casein, edestin, Witte's peptone and deuterio-albumose, inhibit the lytic action of eosin on red cells. Casein lacks glycocoll, gelatin contains about 16%; apparently the presence or absence of this amino acid in the protein molecule is immaterial. The experiments with pure amino acids show definite results. Of those tested, glycocoll, leucin, aspartic acid, α and β alanin, glutamic acid, cystin, phenylalanin and taurin offer no protection while tyrosin and tryptophan are very effective. To a limited extent this coincides with results reported by Hill and Schmidt⁷ on the protection afforded by amino acids to immune bodies against the toxic action of ultraviolet light. There is, however, a striking difference, illustrated in the instance of phenylalanin. The inability of this amino acid to protect against eosin hemolysis shows that the benzene ring alone is not the determining factor. The lack of protection shown by mandelic acid, hydrocinnamic acid and phenylpropionic acid supports this view. Tyrosin, despite its comparative insolubility (1:2,400), and tryptophan⁸ offer marked protection to red cells. The hydroxyphenyl group in a molecule is one of the factors determining ability to protect. This is clearly shown by the fact that hydrocinnamic acid and mandelic acid offer no protection while both orthocumaric and orthohydrocumaric exhibit marked protective ability. Addition of di- and trioxybenzoic acid, resorcin, salicylic acid, pyrogallol or phenol to a mixture of red cells and eosin solution prevents hemolysis on exposure to light.

Since gelatin contains appreciable quantities of prolin (5%), arginin (7.6%), lysin (2.8%) and oxyprolin (6.4%), these amino acids appear to play no part in protecting red cells against the photodynamic effect of eosin. Valin, isoleucin, serin and histidin were not available and no results can be reported. In view of the structure of these amino acids, it appears probable that they are also without influence. The first three do not have a ring structure and histidin lacks the benzene

⁸ This preparation was recrystallized repeatedly and did not give the Millon test.

ring. Little or no protection is afforded by inosite. It must be remembered that although there are six hydroxy groups in this molecule, the substance is a derivative of the reduced benzene ring, hexamethylene, and does not contain a hydroxyphenyl group.

The protection afforded to red cells against eosin hemolysis is not due to the absorption of those light waves that cause eosin to fluoresce. It was found that tubes containing red blood cells and eosin surrounded by a solution of a "protective substance" are hemolyzed in the same time as those surrounded by a water jacket. Tubes containing cells, eosin and a protective substance fluoresce strongly when placed in sunlight; the function of the protective agent does not consist in the absorption of all of the visible fluorescent waves. We have confirmed previous observations that the eosin must be in intimate contact with red blood cells in order to obtain hemolysis. No hemolysis was observed when a suspension of red cells in a quartz test tube was placed in a solution of eosin and exposed to sunlight. Change of acidity is also not a factor concerned in the reaction since no inhibition of hemolysis was observed on addition of a neutral phosphate buffer mixture.

Since photochemical action is conditioned on absorption of light waves, it appears to us that the protection afforded by certain substances is connected with their ability to absorb the active rays and thus prevent their absorption by the red blood cells. If this hypothesis is correct, the toxic action of eosin on protoplasm is determined by the presence of tyrosin or tryptophan in the protein molecules of the cell.

Although our results have shown that certain relations exist between the structure of the protein molecule and ability to protect against the photodynamic effect of eosin, they must nevertheless be considered largely of a qualitative nature. Although certain substances protect red cells against eosin hemolysis, the action is largely dependent on the concentration of eosin, time of exposure and concentration of the protective substance. A number of experiments were also carried out with rose bengal (1:200,000) as photosensitizer. The results obtained were less pronounced than in the eosin series, due no doubt, to the greater potency of this dye in causing hemolysis.

It may be of interest to mention that Bovie⁹ has recently found

⁹ Chem. Engineer, 1919, 27, p. 141.

that in a solution of eosin which had been in contact with thymol for some time and exposed to diffuse daylight, a precipitate in the form of a surface film appeared, indicating that a reaction had taken place between these substances.

THE EFFECT OF THE ADDITION OF CERTAIN SUBSTANCES ON THE HEMOLYSIS OF RED CELLS BY EOSIN

Substance Added	Formula	Concentration	Result on Hemolysis
(a) Proteins:			
Gelatin.....		1%	No inhibition
Blood serum.....		1:5	Complete inhibition
Casein.....		1%	Complete inhibition
Edestin.....		1%	Complete inhibition
Witte's peptone.....		1%	Complete inhibition
Ovomucoid.....		1%	Complete inhibition
Deuteroalbumose.....		1%	Complete inhibition
(b) Amino acids:			
Glycocoll.....	$\text{CH}_2\text{NH}_2.\text{COOH}$	1%	No inhibition
Leucin.....	$(\text{CH}_3)_2:\text{CH}.\text{CH}_2.\text{CH}:(\text{NH}_2).\text{COOH}$	1%	No inhibition
Alanin.....	$\text{CH}_3.\text{CH}(\text{NH}_2).\text{COOH}$	1%	No inhibition
Alanin.....	$\text{NH}_2\text{CH}_2.\text{CH}_2.\text{COOH}$	1%	No inhibition
Aspartic acid.....	$\text{HOOC}.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$	1%	No inhibition
Glutamic acid.....	$\text{HOOC}.\text{CH}_2.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$	1%	No inhibition
Taurin.....	$\text{NH}_2.\text{CH}_2.\text{CH}_2.\text{SO}_3\text{H}$	1%	No inhibition
Cystin.....	$\text{HOOC}.\text{CH}(\text{NH}_2).\text{CH}_2.\text{S}-$	Saturated solution	No inhibition
Phenylalanin.....	$\text{S}.\text{CH}_2.(\text{NH}_2).\text{CH}.\text{COOH}$	1%	No inhibition
Tyrosin.....	$\text{C}_6\text{H}_5.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$	Saturated solution	Complete inhibition
Tryptophan (3 spec.)	$\text{OH}.\text{C}_6\text{H}_4.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$	M/40	Complete inhibition
(c) Other substances:			
Mandelic acid.....	$\text{C}_6\text{H}_5.\text{CHOH}.\text{COOH}$	1%	No inhibition
Hydrocinnamic acid..	$\text{C}_6\text{H}_5.\text{CH}_2.\text{CH}_2.\text{COOH}$	1%	No inhibition
Phenylpropionic acid..	$\text{C}_6\text{H}_5.\text{C}:\text{C}.\text{COOH}$	1%	No inhibition
Inosite.....	$\text{C}_6\text{H}_6(\text{OH})_6$	M/40	Slight inhibition
Ortho-cumaric acid....	$\text{OH}.\text{C}_6\text{H}_4.\text{CH}.\text{CH}.\text{COOH}$	M/200	No inhibition
Ortho-hydrocumaric acid.....		M/40	Complete inhibition
Gallie acid.....	$\text{OH}.\text{C}_6\text{H}_4.\text{CH}_2.\text{CH}_2.\text{COOH}$	M/200	Partial hemolysis
	$\text{C}_6\text{H}_2.(\text{OH})_3.\text{COOH}$ 3:4:5	M/40	Complete inhibition
Pyrogallol.....	$\text{C}_6\text{H}_3.(\text{OH})_3$ 1:2:3	M/200	Complete inhibition
Trioxybenzoic acid....	$\text{C}_6\text{H}_2(\text{OH})_3.\text{COOH}$ 2:3:4	M/1000	Complete inhibition
Dioxybenzoic acid....	$\text{C}_6\text{H}_3.(\text{OH})_2.\text{COOH}$ 2:5	M/200	Complete inhibition
Thymol.....	$\text{C}_6\text{H}_3(\text{CH}_3).\text{OH}.\text{CH}(\text{CH}_3)_2$ 1:3:4	M/40	Complete inhibition
Pbenol.....	$\text{C}_6\text{H}_5.\text{OH}$	Saturated solution	Partial hemolysis
Sodium benzoate.....	$\text{C}_6\text{H}_5.\text{COONa}$	M/40	Partial hemolysis
Salol.....	$\text{C}_6\text{H}_5.(\text{OH})\text{COOC}_6\text{H}_5$ 1:2	1%	No inhibition
		Saturated solution	No inhibition
Glucose.....	$\text{OHCH}_2.(\text{CHOH})_4.\text{CHO}$	1:1000	No inhibition
Cholesterol.....	$\text{C}_{27}\text{H}_{48}\text{OH}$	0.3%	No inhibition
		suspension in 1% gelatin	
Resorcin.....	$\text{C}_6\text{H}_4.(\text{OH})_2$ 1:3	M/200	Complete inhibition
Salicylic acid.....	$\text{C}_6\text{H}_4.(\text{OH}).\text{COOH}$	M/1000	Partial hemolysis
		M/40	Partial hemolysis

SUMMARY

The object of the investigation was to determine the cause of the inability of gelatin to protect red cells against the photodynamic action of eosin. Since gelatin lacks certain amino acids that are contained in the protein molecules which afford protection, experiments were carried out to determine the protection afforded by individual amino acids.

It was found that tyrosin and tryptophan offer marked protection, while phenylalanin, glycocoll, leucin, aspartic acid, alanin, cystin and glutamic acid are ineffective. Certain other substances containing a hydroxyphenyl group in the molecule also protect red blood cells against hemolysis by eosin.

Since action by light cannot take place without absorption, it is possible that the protection afforded to red cells by certain substances against the photodynamic effect of eosin is due to the absorption of the active rays by the protective substance.

THE ANTIGENIC PROPERTIES OF HEMOCYANIN¹

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Comparatively little is known concerning the chemistry of hemocyanin, the copper-protein compound found in the circulating fluid of many Crustacea, Mollusca and other invertebrates. Functionally it appears to play the rôle of oxygen carrier, being in this respect analogous to hemoglobin. Hemocyanin unlike hemoglobin is not confined to particular cells but appears free in the circulating fluid of which in many of these animals it forms the chief protein component.

Concerning its chemical nature no unanimity exists since different workers have obtained results with hemocyanin obtained from different sources which are at variance to an extent greater than usually found in proteins prepared from allied species (1) Henze (2) prepared crystalline hemocyanin from the circulating fluid of the octopus (*Octopus vulgaris*) by precipitation with ammonium sulphate according to the procedure of Hopkins and Pinkus. On dialysis a 3 per cent solution of hemocyanin was obtained. On addition of acid, part, but not all of the copper could be split off, but splitting in the sense that hemoglobin is split to yield a protein and a chromogenic substance did not take place. Henze believes, from a study of its reactions, that hemocyanin is a copper albuminate.

Alsberg and Clark (3) carefully went over the ground covered by Henze but used the blood of *Limulus polyphemus* as a source of hemocyanin. Not only did their product differ from Henze's with respect to the concentration of ammonium sulphate re-

¹ Aided by a grant from the George Williams Hooper Foundation for Medical Research.

quired for precipitation, it behaving in this respect like a globulin, but they also found a marked difference in elementary composition and a decidedly lower copper content. Like Henze, they were unable to obtain from *Limulus* hemocyanin a substance analogous to hematin. It appears that the hemocyanins from these two animals are distinctly different and this conclusion is supported by a comparison of the stability of the oxygen compounds, hemocyanin from *Limulus* being but little dissociated (4) under diminished pressure while that from the octopus gives up its oxygen more readily. Dhéré (5) has also noted differences in the copper content and oxygen capacity of the blood of various invertebrates.

Immunological experiments with pure proteins have shown that antigenic property is intimately connected with their chemical structure. Thus amino acids, polypeptides, protamines, histones, certain proteoses, and gelatin fail to give rise to demonstrable immune bodies when repeatedly injected into animals. Gliadin from wheat and hordein from barley resemble each other in their chemical makeup and are closely related immunologically (6). The immunological method can at times be used for the purpose of gaining further insight into the chemical makeup of a particular protein. Gross chemical analyses yield but little information since they do not take into consideration the stereochemical configuration in the protein molecule, nor is it at times possible by chemical methods to demonstrate differences in closely related proteins which by immunological methods can readily be shown. However it is of interest in this connection to mention that a high histidin content is characteristic of both hemoglobin and hemocyanin (7).

It has been shown that neither globin (8), hematin or their combination, as in hemoglobin (9), is antigenic. The inability of globin to give rise to immune bodies when injected repeatedly into animals cannot be attributed to lack of aromatic amino acids (10) and in this respect differs from gelatin, which, according to an hypothesis (11), is non-antigenic because of this deficiency.

It appeared to us that the immunological method might be used to establish the relationship of the hemocyanins derived from different sources and also to throw further light on its chemical makeup. Nuttall (12) and von Dungern (13) were able to immunize rabbits against the circulating fluid of cephalopods and other invertebrates and von Dungern and Cohnheim (14) found that copper was contained in the specific precipitate obtained with octopus serum, indicating that part of the precipitate consisted of hemocyanin.² While this appears to indicate that hemocyanin is antigenic, it does not exclude the possibility that hemocyanin from other species may not possess this property especially in view of the differences in chemical behavior. Unfortunately for the purpose in mind neither octopus nor limulus blood was available and so no biological comparison could be made. As a source of hemocyanin use was made of the abalone (*Haliotis*) previously also used as a source for taurin (15), and the fluid was obtained in the manner recently described by Myers (16).

The opaque fluid was saturated with oxygen, filtered and an equal volume of saturated ammonium sulphate was slowly added, the fluid being constantly shaken to prevent local zones of high ammonium sulphate concentration. The blue precipitate was filtered off on hardened filter paper, then dissolved in a large volume of distilled water, and saturated ammonium sulphate again added to make a concentration of 4 cc. to each 10 cc. of solution. The precipitate was again filtered off, dissolved as before and reprecipitated by addition of ammonium sulphate to a concentration of 4.3 cc. per 10 cc. of fluid. The precipitate was filtered, redissolved, centrifuged to remove a trace of insoluble matter, and dialyzed first against running and then distilled water, toluol being used as a preservative. The salt-free solution contained a small amount of a white precipitate, probably hemocyanin from which the copper had been split. This was removed by centrifuging. The hemocyanin solution was concentrated by blowing warm dry air over the surface, made isotonic by addition

² These experiments came to my notice after the present work had been completed.

of sodium chloride and for the purpose of injection preserved by the addition of phenol to a concentration of 0.25 per cent. The portion used for the final tests was preserved at a low temperature without addition of preservative.

It will be noted that with respect to its behavior towards ammonium sulphate this preparation of hemocyanin was the same as that obtained by Alsberg from *Limulus polyphemus*. It might also be of interest in passing to mention that in comparison with the hemocyanin content of the fluid, the amounts of albumin and globulin were very small and it is very possible part of these consisted of hemocyanin.

Four normal rabbits were immunized by giving each 8 injections in doses of 80 mgm., a period of five days elapsing between the fourth and fifth injections. Ten days later the animals were bled, the sera inactivated and used for the subsequent tests. Fixation experiments using the well-known hemolytic system in the manner described in previous work with pure proteins (17), gave positive results, the limits of fixation, in terms of serum dilution, being as follows: Rabbit no. 25, 0.1 cc. of 1:250; rabbit no. 27, 0.1 cc. of 1:10; rabbit no. 29, 0.2 cc. of 1:250; rabbit no. 23, 0.4 cc. of 1:50. The usual controls were run to eliminate the possibility of inhibition of hemolysis by factors other than antibody. Positive precipitin tests were likewise given by the above sera. Addition of hemocyanin solution to a suspension of sheep red cells results neither in agglutination nor hemolysis of the red cells.

Three normal guinea-pigs were sensitized by giving each 8 mgm. subcutaneously and five weeks later were reinjected with results as follows: No. 1, 110 mgm. intravenously, death resulted in about ten minutes, symptoms typical of anaphylaxis; no. 2, 100 mgm. intraperitoneally, animal became very sick, temperature dropped 5°C., death resulted six hours after injection; no. 3, 100 mgm. intraperitoneally, slight drop in temperature, no visible symptoms noted.

Hemocyanin, like hemoglobin, is non-toxic. Two guinea pigs were given respectively 75 and 90 mgm. of hemocyanin solution intracardially and a third animal received 105 mgm. intraperi-

toneally. Visible symptoms were not shown and the temperature variation was within the normal variability.

These experiments furnish direct evidence that hemocyanin is antigenic and confirm the previous work of von Dungern and Cohnheim. Its chemical makeup must be very different from hemoglobin since the latter is non-antigenic.

SUMMARY

1. Hemocyanin prepared from the circulating fluid of the abalone (*Haliotis*) was precipitated by ammonium sulphate within the limits found by Alsberg and Clark from *Limulus polyphemus*. It appears to be a globulin.

2. The sera of rabbits immunized with this preparation of hemocyanin gave positive fixation and precipitin tests. It was toxic for guinea-pigs previously sensitized to this substance but not toxic for normal animals.

3. These experiments support the chemical viewpoint that the chemical makeup of hemocyanin is very different from hemoglobin since the latter is non-antigenic.

REFERENCES

- (1) KOSSEL, A., AND KUTSCHER, F.: Z. physiol. Chem., 1900-1901, **6**, 31, pp. 165-214, have shown that considerable differences exist in the amino acid content of salmin, clupein, cyclopterin, and sturin. Nevertheless these substances give the general reactions characteristic of the protamines. The differences in composition of the proteins of horse and ox serum noted by HARTLEY, P., Biochem. J., 1914, **8**, pp. 541-552, are not striking, and GORTNER, R. A., AND WUERTZ, A. J., J. Amer. Chem. Soc., 1917, **39**, pp. 2239-2242, find that within the experimental error the composition of fibrin obtained from various animals is the same.
- (2) ⁵/₄ HENZE, M.: Z. f. physiol. Chem., 1901, **33**, pp. 370-384.
HENZE, M.: Z. f. physiol. Chem., 1904, **43**, pp. 290-298.
- (3) ALSBERG, C. L., AND CLARK, E. D.: J. Biol. Chem., 1910, **8**, pp. 1-8.
ALSBERG, C. L.: J. Biol. Chem., 1914, **19**, 77-82.
- (4) ALSBERG, C. L., AND CLARK, W. M.: J. Biol. Chem., 1914, **19**, 503-510.
- (5) DHÉRE, C.: J. Physiol. et Path. gen., 1915, **16**, 985-997.
DHÉRE, C.: J. Physiol. et Path. gen. 1919, **18**, 222-243.
- (6) WELLS, H. G., AND OSBORNE, T. B.: J. Infect. Dis., 1911, **8**, 66-124.
- (7) VAN SLYKE, D. D.: J. Biol. Chem., 1911-12, **10**, 15-55.

- (8) GAY, F. P., AND ROBERTSON, T. B.: J. Exp. Med., 1913, **17**, 535-541.
SCHMIDT, C. L. A.: Univ. Cal. Pub. Path., 1916, **2**, 157-204.
BROWNING, C. H., AND WILSON, G. H.: J. Path. & Bact., 1909, **14**, 174-183.
- (9) FORD, W. W., AND HALSEY, J. T.: J. Med. Res., 1904, **11**, 403-425.
LEVENE, P. A.: J. Med. Res., 1904, **12**, 191-194.
SCHMIDT, C. L. A., AND BENNETT, C. B.: J. Infect. Dis., 1919, **25**, 207-212.
BRADLEY, H. C., AND SANSUM, W. D.: J. Biol. Chem., 1914, **18**, 497-506.
- (10) SCHITTENHELM, A., AND WEICHARDT, W.: Z. Immfrsch. & exp. Therap. Orig., 1912, **14**, 609-636.
- (11) WELLS, H. G.: J. Amer. Med. Assoc., 1908, **50**, 527-528.
- (12) NUTTALL, G. H. F.: Blood Immunity and Blood Relationship, Cambridge, 1904, p. 358.
- (13) VON DUNGERN, E.: Centralbl. Bakt. Ref., 1904-1905, **35**, 253.
- (14) VON DUNGERN, E., AND COHNHEIM, O.: Die Antikörper, Jena, 1903.
- (15) SCHMIDT, C. L. A., AND WATSON, T.: J. Biol. Chem., 1918, **33**, 499-500.
- (16) MYERS, R. G.: J. Biol. Chem., 1920, **41**, 119-135.
- (17) SCHMIDT, C. L. A.: Univ. of Cal. Pub. Path., 1916, **2**, 157-204.

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INTOXICATION OF INTESTINAL OBSTRUCTION

TOXIC PROTEOSES NOT DESTROYED IN INTESTINAL TRACT AND
NOT FORMED IN COLON LOOPS

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MUCH has been written about the interesting type of intoxication which develops with an intestinal obstruction. The fact that many explanations have been championed by different investigators and clinical observers is sufficient proof that convincing evidence is not at hand to explain all the observed reactions. We wish to point out several observations and add other experiments which bear on this subject. We believe that some of these facts have been overlooked by some of the recent investigators and that a proper grasp of these data will clear the field of many confusing and apparently contradictory theories.

It is now admitted by practically all investigators in this field that a poison is formed in the body and is responsible for the intoxication which develops. Where this poison develops, what its nature may be, and how it is formed are questions open to debate and have been discussed with much earnestness and even polemical vigor by the many workers who are seeking the solution of this problem. That this intoxication is purely "non-specific" gives it an added interest to many workers, and makes it probable that information gained concerning this intoxication may have value in the proper understanding of the "non-specific" fraction of intoxications associated with bacterial infection.

One most important point has been lost sight of and needs constant emphasis—*Nothing produced within the lumen of the intestinal tract can be directly concerned in the intoxication of intestinal obstruction*, because the intestinal epithelium is impervious to all toxic substances which can be demonstrated in any amount in the material accumulating in the obstructed intestine. Material

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obtained from the obstructed intestine can be fed in unlimited quantities or injected into the normal duodenum or obstructed intestine, or into closed loops of the intestine without causing the slightest degree of intoxication. These facts have been demonstrated beyond any doubt in published experiments (1, 2) and the data given below add confirmatory evidence. Therefore we need not concern ourselves with hypothetical toxins which may be produced in the lumen of the obstructed intestine by bacterial growth or by ferment cleavage of protein substances. There is no evidence that any such toxins are formed under the condition of intestinal obstruction, but if they are formed they cannot be concerned in the general intoxication, because these substances cannot be taken up by the intact intestinal mucosa.

We believe that the epithelium of the mucous membrane of the small intestine under obstruction conditions is able to form a toxic substance or substances, and that this poison can be formed only by the intestinal epithelium. Unusual development of intestinal bacteria may act as a stimulant, but the essential thing is that the toxic substance must be *formed in the mucous membrane*. As the poison is being formed in the mucosa of the small intestine a part of it is taken up by the blood stream and causes the characteristic clinical reaction, a part is not taken up, but is excreted into the lumen of the gut, and once in the intestinal lumen the poison is *inert* in so far as the host is concerned. This portion of the poison may be obtained from the material in the lumen of the obstructed intestine. It is to be kept in mind that this poison is not found in the normal intestine.

The substance or substances which we have isolated from obstruction material have been described in other communications (3) (4). It is sufficient to say that the evidence points to the proteose group of substances, and we shall use the term "proteose" with proper mental reservation to indicate the possibility of other large molecular substances being present in the "proteose mixture."

EXPERIMENTAL OBSERVATIONS

Closed Loop of Colon. Duration Ten Months. Dog 18-8. Collie. Healthy Female. Weight 25 pounds. July 19, 1917. A closed loop of colon done in the usual way by Dr. Woolsey and Dr. Kerr. The loop included about

3 inches of the ileum and all of the colon up to about 5 inches from the anus. The ends were cut across and inverted. A lateral anastomosis was made between the ileum and rectum to re-establish the intestinal tract.

July 23d to 31st. Uninterrupted convalescence. Weight 25 pounds.

Oct. 3d. Dog is normal and lively. Weight 29.5 pounds. Isolated in metabolism cage without food.

Oct. 4th. A small amount of fecal material removed from cage and discarded.

3 P.M. *A solution of toxic proteose, 200 c.c., given by stomach tube.* Obtained from autopsy 17-60, described below.

Oct. 5th. 10 A.M. Dog normal in every respect. No feces.

Again given 200 c.c. of a solution of toxic proteose. Autopsy 17-60. There is no clinical reaction following either administration of proteose.

2 P.M. Soft yellow semi-fluid feces passed and immediately collected from cage. Weight 50 grams.

Fecal material dissolved in water (about 100 c.c.), separated by centrifuge and the supernatant fluid precipitated by five volumes of 95 per cent alcohol. This alcoholic precipitate dissolved in distilled water, slightly acidified and boiled, then filtered to remove a small amount of precipitate. This neutralized watery extract of the alcoholic precipitate was shown to contain much toxic proteose-like material tested on Dog 18-57. This dog was severely but not fatally intoxicated. See below.

Oct. 6th, 10 A.M. Soft semi-fluid yellow *feces*, 30-40 grams in weight, collected carefully and extracted for proteose exactly as described for preceding day. This material tested on Dog 18-58 gave a lethal and typical intoxication, fatal within five hours. The autopsy findings were typical of acute intoxication following the intravenous injection of proteose material obtained from cases of intestinal obstruction.

12 M. Dog given 200 c.c. milk by stomach tube. Dog normal in all respects.

Oct. 7th. No feces passed.

Oct. 8th. 10 A.M. Soft semi-fluid yellow *feces*, 50 grams in weight, collected as usual and extracted for proteose-like material exactly as described above. This extract was tested upon Dog 18-55 and was shown to contain no toxic material whatsoever. (*Control period.*)

Mar. 11, 1918. Dog 18-8 in normal condition. Weight 30.8 pounds.

May 15th. Dog is not in good condition and shows evidence of definite intoxication. Abdomen is prominent. Weight 32 pounds.

May 17th. Dog is very sick. Abdomen distended. Vomiting. Ether anesthesia and sacrifice.

Autopsy performed at once. Thorax, heart, and lungs normal. Peritoneal cavity contains 3000 c.c. turbid straw-colored fluid with a sediment of granular material, the granules of fibrin measuring 2 to 3 mm. in diameter. Serous surfaces are injected and swollen, the omentum particularly. Stomach contracted and normal; small intestines collapsed and normal throughout; the anastomosis between the ileum and colon is imbedded in a mass of dense adhesions which contain some pus pockets. This long latent period is of some surgical interest. These foci of infection undoubtedly explain the general peritonitis. Spleen is somewhat enlarged and mottled. Liver pale and yellow and contains two superficial small pus pockets just beneath the capsule anteriorly. This is the result of an extension of the inflammatory process from the peritoneum into the parenchyma. Pancreas and kidneys are normal.

Loop of Colon. Its walls are definitely hypertrophied, particularly in the short stump of the ileum. There are very few adhesions over the serous surfaces. The mesentery is thick and tendinous. Mucous membrane is normal, perhaps a little thickened. The inverted ends are clean and the most careful examination shows no ulcers anywhere. The ileum measures 3 inches in length and contains only traces of yellowish fecal-like material. A large sausage-like mass of material was packed into the lower colon. This material is putty-like in consistency and of a dark slaty color. It separates very easily and cleanly from the mucous membrane. It is homogeneous throughout.

Microscopical sections in general are negative. Liver shows a little central fatty degeneration. Sections from the stomach, intestines and various parts of the closed loop show normal epithelium everywhere.

Material from closed loop weighs 126 grams. It was ground up in about 250 c.c. of distilled water, sand being added in the mortar. It was reduced to a thin soupy fluid and then shaken for hours in the shaking-machine. All particles were thrown down by the centrifuge, giving a supernatant fluid of opalescent gray appearance. This was precipitated with five volumes of 95 per cent alcohol. After standing for two days the alcoholic precipitate was dissolved in water (700 c.c.), made slightly acid to litmus with acetic acid, brought to a boil and filtered. The filtrate was concentrated over a water-bath after neutralization to 100 c.c. All of this material was given intravenously to Dog 19-19. There were no evidences of intoxication. See below.

Toxic Proteose from Case of Human Obstruction, Autopsy 17-60. Young adult male; operation upon lower ileum. Shortly after this operation evidences of intestinal obstruction developed with very rapid intoxication and death. Autopsy was performed within two to three hours

post-mortem. A kink in the ileum was found just above the enterostomy wound.

Intestinal fluid was obtained in large amounts from the distended ileum. This material had the usual appearance and odor characteristic of such material. It was cleared by the centrifuge and the watery broth-like fluid material was poured into five volumes of 95 per cent alcohol. After several days the alcoholic precipitate was taken up in distilled water, acidified with acetic acid, brought to a boil, and filtered. This clear amber filtrate was neutralized and tested out on a normal dog, 18-43. Weight 27 pounds. Ten c.c. per pound body weight given intravenously gave a very severe, almost fatal, intoxication with repeated vomiting, bloody diarrhea, and prostration. This material (4000 c.c. in all) was then concentrated to 2000 c.c., autoclaved, and preserved. It is obvious that 5 c.c. per pound body weight should approximate a lethal dose. This human material, containing much of the characteristic poison, was given by stomach tube in considerable doses to the dog 18-8, used in the first experiment.

Proteose Recovered from Feces after Administration by Mouth. Dog 18-57. Fox-terrier, adult female. Weight 24.3 pounds.

Oct. 29th, 12 M. Ether anesthesia and intravenous injection of *proteose extract obtained from Dog 18-8; fecal material of Oct. 5th.* 200 c.c. total amount of deep amber-colored fluid. This injection caused little depressant effect on blood pressure.

12:40 P.M.—Temperature 38.3. Passed solid feces.

2:00 —Temperature 38.0.

2:30 —Temperature 38.4. Diarrhea and vomitus.

3:20 —Temperature 38.8. Dog very sick. Bile-stained vomitus.

3:50 —Temperature 39.1. Continuous vomiting and diarrhea.

4:45 —Temperature 39.2. Vomiting continuous.

5:30 —Temperature 39.1. Clinical improvement.

Oct. 30th: Dog appears well.

This experiment shows that toxic proteose material from a case of intestinal obstruction, fed to a dog with excluded large intestine, may be recovered unchanged from the feces. There is no resultant intoxication from feeding this poison. The toxic proteose is able to resist the action of the digestive enzymes of the stomach and small intestine for a period of forty-eight hours or longer. We have reported (5) experiments to show that this toxic substance is able to resist long periods of digestion *in vitro*.

Proteose Isolated from Feces after Administration by Mouth. Dog 18-58. Fox-terrier, small adult male. Weight 12.8 pounds.

Oct. 29th, 12 M. Ether anesthesia and intravenous injection of *proteose extract obtained from Dog 18-8. Fecal material of Oct. 6th.* 150 c.c. total amount of amber-colored opalescent fluid.

12:40 P.M.—Temperature 38.0. Mucous diarrhea.

2:00 —Temperature 38.0. Mucous diarrhea and bile-stained vomitus.

2:30 —Temperature 37.7. Vomitus and diarrhea, prostration.

3:15 —Temperature 37.6. Prostration. Pulse very weak.

3:50 —Temperature 36.8. Condition unchanged.

4:50 —Temperature 36.4. Profound intoxication.

5:20 —Death and autopsy immediately.

Blood obtained in oxalate solution showed practically no plasma on standing. This blood concentration is very common in severe proteose intoxication. Thorax, heart, and lungs normal. Liver, spleen, pancreas, and kidneys are swollen and engorged with blood. Stomach shows a pale pyloric mucosa and pink swollen cardiac mucosa. Mucous secretion is very abundant. Duodenum, jejunum, and ileum present a deep purple velvety mucosa coated with a thick layer of mucus. Colon shows definite engorgement. This *autopsy is in every way typical* of acute intoxication resulting from a lethal dose of the proteose obtained from an obstructed intestine.

This experiment confirms in every respect the preceding observation. There seems to have been little destruction or loss of the toxic proteose and its toxicological reaction is identical with that of the original material before passage through the intestinal tract. Such resistance toward the digestive enzymes is remarkable.

Proteose Absent from Feces. Control Experiment. Dog 18-55. Spaniel, small adult female. Weight 19.3 pounds.

Oct. 22, 11 A.M. Ether anesthesia and intravenous injection of *proteose extract obtained from Dog 18-8. Fecal material of Oct. 8th.* 120 c.c. total amount obtained as described above. This injection caused a transient fall in blood pressure, but no change in the temperature curve. There were no clinical signs of intoxication at any time.

These control feces contained no toxic material in any way comparable to the material isolated and tested in the two preceding experiments. These feces were collected from the same dog, 18-8, with the exception that milk was given by stomach tube instead of the proteose solution. This experiment gives the necessary control

to the two preceding observations and substantiates other observations to show that the normal intestinal tract contains no toxic proteose material.

Proteose Isolated from Colon Loop Material Non-toxic. Dog 19-19. Mongrel, adult male. Weight 23.3 pounds.

Aug. 13th, 12 M. Ether anesthesia and intravenous injection of *proteose extract obtained from the colon loop* material of Dog 18-8. The entire proteose extract made as described above, concentrated to 100 c.c., given slowly intravenously. This injection caused only a slight fall in blood pressure with considerable flushing of the skin. It caused no temperature reaction of any appreciable degree and not the slightest evidence of clinical intoxication.

Compare with the several experiments given just below to show that these colon loops are non-toxic and do not form toxic proteose-like substances.

Closed Loop of Colon. Duration Four Months. No Intoxication. Dog 17-111. Airedale, young adult female. Weight 22 pounds.

Jan. 3d. Dr. Woolsey isolated a *closed loop of the colon* as described above and established the intestinal tract by anastomosis between the ileum and the rectum.

Jan. 5th to 8th. Normal recovery. Weight 19.3 pounds.

Feb. 2d. Dog normal and regaining lost weight. Weight 21.8 pounds.

May 1st. Dog has been uniformly in good condition. Weight 27.8 pounds. There has been no evidence of any intoxication at any time. Dr. Woolsey and Dr. Kerr *excised the loop of colon* with no operative difficulty.

Loop of Colon includes 1 inch of ileum which is empty. The cecum is empty. The lower end of the colon loop contains a large sausage-like mass which has the general appearance and consistency of normal feces. It is uniform and pasty throughout and separates cleanly from a normal pale mucous membrane. The lower end of the colon where inverted shows one tiny ulcer, not over 2 mm. in diameter. Without exception the mucosa elsewhere is pale, normal, and intact. The microscope shows a normal mucous membrane except for the tiny superficial ulcer described above. Goblet cells and mucus are conspicuous.

Colon Loop Material (about 150 grams in weight). It was ground with water and sand to a thin soupy mixture, the final volume about 400 c.c. This was not centrifuged, but poured direct into five volumes of 95 per cent alcohol. The alcoholic precipitate dissolved in water (about 1200 c.c.)

slightly acidified with acetic and brought to a boil. The precipitate was removed by means of the centrifuge, giving a grayish opalescent fluid somewhat like clam broth. This material was neutralized and tested out upon a number of normal puppies.

Material from Closed Loop of Colon. Dog 17-111. Tested by intravenous injection upon the following animals:

Pup 17-207. Weight 14 pounds. 2 c.c. per lb. intravenously. No intoxication.

Pup 17-181. Weight 14.3 pounds. 7 c.c. per lb. intravenously. No intoxication.

Pup 17-217. Weight 10.3 pounds. 8 c.c. per lb. intravenously. Slight intoxication.

This last experiment (Pup 17-217) showed a little clinical intoxication and slight febrile reaction. The amount injected was very large for a small puppy, but it may indicate a trace of toxic material in this closed loop.

Proteose Absent in Feces after Colon Extirpation. Dog 18-42. Fox-terrier, adult female. Weight 12.8 pounds.

Sept. 27th, 12:30 P.M. Ether anesthesia and intravenous injection of *proteose extract obtained from feces of Dog 17-111*. Thin yellow feces (123 grams in weight) obtained fresh from cage, ground up and extracted with 400 c.c. distilled water, agitated in shaking-machine for five hours and separated by centrifuge. The opalescent yellowish broth-like fluid poured into five volumes of 95 per cent alcohol. After two days the alcoholic precipitate extracted as usual with water, acidified, brought to a boil, filtered, and concentrated over the water-bath to 150 c.c. The final solution was almost clear, pale amber in color. Total amount of this extract was given intravenously with definite but moderate depression of the blood pressure. There was a slight febrile reaction, but no definite clinical shock and no signs of the usual intoxication.

This experiment gives confirmatory data to show that toxic proteoses are not present in the small intestine of the dog. The colon extirpation makes the collection of soft feces very easy and excludes the possibility of any neutralizing action taking place in the colon.

Discussion. The experiments outlined above are capable of but a single interpretation. It is quite clear from these experiments and other similar observations which need not be recorded at this time, that closed colon loops are never associated with any definite clin-

ical intoxication referable to the closed loop. This is very different from closed loops of the small intestine which invariably are associated with distinct evidences of intoxication. Closed loops of the colon cause no disturbance even if isolated for many months (four to ten or longer). Material accumulates slowly in these loops, and this material looks remarkably like normal fecal material. It is made up mainly of cell debris and masses of bacteria. No toxic proteose material can be isolated from this colon loop material. Again, this differs from the material which accumulates in a closed loop of small intestine as such material is rich in toxic proteose. It is obvious, therefore, that the colon cannot take any active part in the intoxication of intestinal obstruction.

The proteose material isolated from an obstructed intestine or closed intestinal loop is known to be resistant to digestion *in vitro* (5). These experiments show conclusively that this same proteose material can resist the digestive enzymes of the intestinal tract of the dog for forty-eight hours or longer. It is possible to recover this proteose from the feces after its administration by stomach tube.

These experiments give more evidence to prove that these toxic proteoses are not present in the small intestine of the normal dog. A study of the fecal material obtained from dogs whose colons have been removed shows that a toxic proteose is not present in the small intestine and excludes any neutralizing action on the part of the colon.

BIBLIOGRAPHY

1. Whipple, Stone, and Bernheim, *J. Exper. M.*, 1914, XIX, 166.
2. Davis, D. M., *Johns Hopkins Hosp. Bull.*, 1914, XXV, 33.
3. Whipple, Rodenbaugh, and Kilgore, *J. Exper. M.*, 1916, XXIII, 123.
4. Whipple and Van Slyke, *J. Exper. M.*, 1918, XXVIII, 213.
5. Whipple, Stone, and Bernheim, *J. Exper. M.*, 1913, XVII, 307.

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I. RAPID BLOOD PLASMA PROTEIN DEPLETION AND THE CURVE OF REGENERATION

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The published work of Kerr, Hurwitz and Whipple (1) brings out several facts about the blood serum proteins which may be mentioned briefly before we go on to a consideration of the experiments given below. The *stability of the serum protein concentration* is truly remarkable and obviously of some importance to the body physiology. The normal level is quite constant and considerable deviations from this normal base line are not well tolerated by the body; in fact, profound shock may result. When the serum proteins are depleted or washed out by the technique employed the repair or regeneration of these proteins is a slow process requiring from 5 to 10 days, depending upon the amount removed and other factors. It is as difficult to reconstruct these proteins as it is for the body to repair and replace liver cells following an extensive liver injury. It appears that the liver is especially concerned in the normal regeneration of blood serum proteins. Fasting does not prevent serum protein regeneration, therefore it is possible for the body to release these substances or to construct serum proteins from its own protein end products. There is no evidence that increased nitrogen breakdown is responsible for this regeneration of serum protein.

The experiments of Kerr, Hurwitz and Whipple were different as to method of blood serum depletion when compared with the experiments given below. These earlier experiments were mostly done by *interval bleedings* followed in each instance by a return of the washed red corpuscles suspended in modified Locke's solution. Under such circumstances a dog was bled 100 to 200 cc. and after washing by centrifugalization the same red cells were returned intravenously in Locke's solution. This procedure was repeated several times during the day until the serum protein depletion was carried to a minimum figure.

This method has been used by Abel, Rowntree and Turner (2) and called "plasmapheresis." It is obvious that this experimental procedure introduced wide fluctuations in blood volume and it was suspected that the shock which resulted was to be explained by the repeated hemorrhages and infusions.

We shall use the term *plasma depletion* or *plasmapheresis* to indicate a removal of plasma proteins by means of repeated hemorrhage followed or accompanied by the replacement of like amounts of red cells suspended in a protein-free fluid. The plasma depletion in our experiments was effected by a method first introduced by Morawitz (3); bleeding and the removal of whole blood was simultaneous with the replacement of the red cell Locke's solution mixture. The inflow and outflow volume was at all times constant and obviated any fluctuation in blood volume. All aseptic precautions were taken in manipulation, washing and final preparation of the red cell mixtures. The red cell mixtures were introduced at body temperature and the dog was kept warm during the experiment.

The method employed in these experiments enables an investigator to reduce the blood plasma proteins from the normal level of 5 to 6 per cent to a very low level of 1.5 to 0.9 per cent. This can be done in a matter of minutes (2 to 10 minutes) leaving the animal uninfluenced by the large and numerous fluctuations in blood volume and oxygen-carrying capacity of the blood which undoubtedly occur in the method used by Kerr, Hurwitz and Whipple. In addition it facilitates observations of that portion of the curve of protein regeneration immediately following a large single depletion and permits observations on an uninterrupted regeneration curve.

METHOD

The animals used were sound young dogs maintained on a mixed diet. In most cases no food was given the animal for a period of 12 hours preceding the experiment. Free access to water obtained. Under complete ether anesthesia and with aseptic precautions an incision was made either into the region of the femoral vessels or of the large vessels of the neck. The artery and vein were exposed and clamped. Into each was introduced a vaseline-coated cannula pointing toward the heart. Plasma removal was effected by withdrawing through the cannula placed in the artery large quantities of blood. This blood was allowed to flow into a graduated bottle. Simultaneously, a suspension

of washed corpuscles warmed to body temperature was injected under pressure through the venous cannula. This suspension was delivered from a flask which was also graduated. The graduations were used to permit a comparison of the inflow with outflow to be made at any time during the exchange. In this way inflow and outflow were observed and kept equal at all times. In order to maintain an even suspension of the injection mass, the latter was frequently shaken.

The fluid injected consisted of washed dog corpuscles suspended in a modified Locke's solution in the ratio of three parts packed corpuscles to two parts by volume of the saline mixture. The composition of the Locke's solution was: sodium chloride, 0.9 per cent; potassium chloride, 0.042 per cent; sodium bicarbonate, 0.02 per cent. The corpuscles in all cases were obtained from the blood of healthy dogs. This blood was drawn into sodium oxalate, centrifugalized, and the plasma removed from the sedimented corpuscles. The corpuscles were then washed twice in the modified Locke's solution by resuspension and centrifugalization. Aseptic precautions were observed in all these manipulations.

The exchange was effected in a period ranging in different animals from 2 to 25 minutes. The amount of fluid withdrawn varied in individual experiments from 60 per cent to 195 per cent of the animal's blood volume. The amount of blood simultaneously injected corresponded within a few cubic centimeters to the amount withdrawn. The blood volume was estimated as 10 cc. per 100 grams of body weight. In a number of experiments the actual blood volume was kindly determined for us by Dr. C. W. Hooper, using a dye method recently described. This paper (4) shows that the blood volume of active normal dogs as determined by the dye method is approximately 10 cc. per 100 grams of body weight.

At the end of the operative procedure the cannulae were withdrawn and the vessels ligated. Vaseline was applied liberally to the wound. In a few instances it was necessary to make a single suture through the subcutaneous tissues at the site of operation. In practically every experiment the wound healed quickly with little or no suppuration.

Samples of blood were collected through the arterial cannula at the beginning and at the end of the exchange and again 15 minutes later. Subsequent samples were taken from the jugular vein by means of a needle and syringe. On each of these occasions two samples were withdrawn. One of these was drawn into a 15 cc. hematocrit tube containing 3 cc. of 1 per cent sodium oxalate. The other sample was drawn into a plain heavy-walled glass test tube and allowed to clot. Both

of these samples were then centrifugalized at a high rate of speed. The serum from the clotted sample was then used for estimation of serum albumins, serum globulins and the non-protein fraction by the refractometric method of Robertson (5). Percentage corpuscles readings were made from the oxalated sample, correction being made for the amount of oxalate solution present. The plasma from this sample was also used for the determination of fibrin. This was carried out according to the method of Cullen and Van Slyke (6). This method consists in diluting 5 cc. of plasma in 100 cc. of salt solution. To this mixture 1.5 cc. of a 2.4 per cent solution of CaCl_2 was added to supply calcium and to promote clotting, and a Kjeldahl done on the mass of fibrin obtained.

The clinical condition of the animal was closely observed. The rectal temperature, rate of respiration, pulse, diarrhea and vomitus, as well as the general appearance of the animal, were noted. In cases in which death resulted, careful autopsies were performed at once. The clinical condition of the animal will be made the subject of a subsequent paper with a discussion of the peculiar type of shock which may develop under these conditions.

EXPERIMENTAL OBSERVATIONS

This paper in general deals with the recovery experiments but in certain tables we include many of the lethal shock experiments (tables 12, 13 and 14). For the sake of comparison we give in table 1 a type experiment which was followed promptly by fatal shock. Many of these experiments will be found in the next paper of this series and in that place the general discussion of this peculiar shock will be presented. It will be noted in table 1 that the reduction of total proteins, albumin and globulin is pretty uniform and is a fall to approximately one-third of normal. The emergency increase of protein during the 15 minutes following the plasma depletion is not as marked as usual (see table 2). There is no further increment of serum protein in the hour following this 15-minute sample and this may be explained in part by the profound shock. The content of red cells in whole blood as shown by the hematocrit is lower than usual and the fall which appears immediately after plasmapheresis would indicate the use of a red cell mixture containing fewer red cells than intended. This factor does not complicate the remaining experiments and we believe has no significance.

Experiment 104. (See table 1). 122 per cent exchange.

Dog 18-48. Young female bull dog. Weight 16.9 pounds. Blood volume on November 20, 1917 (by dye method) was 941 cc.

November 20, 1917. Under ether anesthesia 940 cc. blood were withdrawn from the left carotid artery. Simultaneously 1000 cc. of blood corpuscle suspension were injected into the left external jugular vein. The duration of the exchange was 10.5 minutes. Animal showed almost immediately a great fall in pulse pressure and arterial tension. Profound depression with forced irregular respiration developed in about an hour. Death 2 hours after the exchange. The autopsy findings are uniform in all fatal experiments and will be described in detail in the following paper.

TABLE 1

122 per cent blood volume exchange; dog 18-48; experiment 104

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATO- CRIT RED CELL PER CENT	REMARKS
	Total protein	Albu- min	Globu- lin	Non- protein			
Before exchange.....	5.7	4.2	1.5	2.0	0.25	47	
Immediately after...	1.9	1.4	0.5	1.6	0.12	35	Considerable hemolysis
15 minutes after.....	2.3	1.9	0.4	1.7	0.11	38	Considerable hemolysis
1 hour and 20 min- utes.....	2.4	2.0	0.4	2.1		30	Fatal shock

Table 2 gives the results of an experiment which contrasts with this lethal shock experiment (table 1). The second experiment presents an even large blood exchange in plasmapheresis but this dog is not disturbed by the procedure. It will be noted that there are marked individual differences in dogs as to their tolerance to this plasma depletion. Any given dog will show a considerable uniformity of reaction to a unit exchange but must be standardized to ascertain this reaction. Table 2 also shows a fall of total protein, albumin and globulin to about one-third normal following the plasmapheresis. We wish to call attention to the emergency increase in blood proteins which appears *within 15 minutes*. This is a characteristic reaction which obtains in practically all experiments (tables 11 and 12). The increase in serum proteins during the next 24 hours is very marked and exceeds 1 per cent protein—equivalent to more than 20 per cent of the total protein replaced in the blood serum. Subsequent regeneration of protein in the serum is slow and requires several days for complete recovery.

Experiment 69. (See table 2). 170 per cent exchange.

Dog 18-9. Young female bull dog. Weight 14 pounds. Blood volume on July 19, 1917 (by dye method) was 772 cc.

August 2, 1917. Under ether anesthesia 1081 cc. blood were withdrawn from the right femoral artery. Simultaneously 1081 cc. of blood corpuscle suspension were injected into the right femoral vein. The duration of exchange was 12 minutes. Following the exchange the temperature fell about 2 degrees, but returned to the original level within 2 hours. Pulse and respiration were fair at all times.

A much smaller volume exchange is shown in table 3, yet considerable shock resulted. It will be seen that the level of total proteins, albumin and globulin falls to approximately one-half of normal corresponding to the smaller exchange volume (90 per cent). There is a

TABLE 2

170 per cent blood volume exchange; dog 18-9; experiment 69

TIME	BLOOD SERUM READINGS IN PER CENT				REMARKS
	Total protein	Albumin	Globulin	Non-protein	
Before exchange.....	5.6	3.8	1.8	1.7	
Immediately after.....	2.0	1.3	0.7	1.4	Moderate hemolysis
15 minutes after.....	2.9	2.0	0.9	1.3	Moderate hemolysis. No shock
2d day.....	4.2	3.1	1.1	1.9	Slight hemolysis. Normal
3d day.....	4.5	2.5	2.0	2.1	
4th day.....	4.8	3.2	1.6	1.7	

moderate increase in serum proteins during the 15-minute period and less than usual during the first 24 hours following plasmapheresis. We cannot explain satisfactorily the remarkable drop in red cell hematocrit which is present after 11 hours and persists many days. Possibly the red cells used for infusion in this experiment had been seriously injured and went to pieces in the circulation. In confirmation of this suggestion we note the presence of hemolysis in blood samples taken on the first four days following the experiment. It may be suspected that an hemolysin was present in this dog's blood but there is reasonable doubt whether hemolysins actually do occur in the dog in sufficient amount to destroy large numbers of homologous red cells.

Experiment 103. (See table 3). 91 per cent exchange.

Dog 18-66. Young female bull mongrel. Weight 17 pounds.

November 15, 1919. Under ether anesthesia 700 cc. blood were withdrawn from the right femoral artery. Simultaneously 700 cc. of blood corpuscle suspension were injected into the right femoral vein. The duration of exchange was 9 minutes. Following the exchange the temperature showed little or no alteration from the original level. The pulse was regular but poor in tension for a number of hours. One-half cubic centimeter of adrenalin subcutaneously was given 4 hours after the operation. After 24 hours the animal was in excellent condition.

In all these experiments the washing out of plasma proteins is accomplished by a *rapid exchange*. The bleeding and simultaneous infusion of the red cell mixture occupies only a few minutes, the limits being

TABLE 3
91 per cent blood volume exchange; dog 18-66; experiment 103

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATO-CRIT RED CELL PER CENT	REMARKS
	Total protein	Albumin	Globulin	Non-protein			
Before exchange.....	6.2	4.5	1.7	2.0	0.42	49	
Immediately after...	3.2	2.2	1.0	1.5		49	
15 minutes after.....	3.8	2.9	0.9	1.6	0.21	56	
3 hours.....	4.3	3.0	1.3	1.7	0.58	50	Moderate shock
11 hours.....	4.0	2.9	1.1	2.3	0.47	33	
2d day.....	4.3	2.8	1.5	2.6	0.48	35	Dog normal. Hemolysis
3d day.....	4.8	3.7	1.1	2.5	0.47	29	Hemolysis
5th day.....	4.5	3.1	1.4	3.0	0.41	27	Hemolysis
6th day.....	5.3	3.9	1.4	2.3			
8th day.....	5.4	3.8	1.6	2.9	0.45	32	
10th day.....	5.7	4.1	1.6	2.0	0.42	32	
12th day.....	5.4	3.9	1.5	2.1	0.56	32	

2 to 25 minutes. Within these limits the speed of exchange, whether 2 minutes or 25 minutes, seems to make little difference. To make this point clear we may contrast tables 4 and 5. The first of these two experiments done on the same animal (table 4) shows the reaction following an exchange of 100 per cent done in 14 minutes. There was definite shock but a rapid recovery. The second experiment done on this dog after an interval of 2 weeks to insure complete recovery, shows the reaction following a very rapid 100 per cent exchange which was completed *within 2 minutes*. There was if anything less shock on this occasion than after the first exchange. It is interesting to note how closely

the curves of total protein, albumin and globulin in the two experiments coincide. The prompt rise in the 15-minute interval is identical and the initial fall corresponds to the other experiments discussed.

Experiment 93. (See table 4). 99 per cent exchange.

Dog 18-48. Young female bull dog. Weight 13.3 pounds.

October 4, 1917. Under ether anesthesia 600 cc. blood were withdrawn from the right femoral artery. Simultaneously 700 cc. of blood corpuscle suspension

TABLE 4
99 per cent blood volume exchange; dog 18-48; experiment 93

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATO-CRIT RED CELL PER CENT	REMARKS
	Total protein	Albumin	Globulin	Non-protein			
Before exchange.....	4.5	3.2	1.3	2.2	0.26	58	Definite shock
Immediately after....	2.0	1.3	0.7	1.6	0.16	58	
15 minutes after.....	3.3	2.6	0.7	1.5	0.15	58	
9½ hours.....	3.9	3.0	0.9	1.8	0.22	46	Normal
2d day.....					0.18	54	
3d day.....	4.0	3.1	0.9	1.9	0.30	42	
4th day.....	4.4	3.3	1.1	1.7	0.39		

TABLE 5
104 per cent blood volume exchange; dog 18-48; experiment 96

TIME	BLOOD SERUM READINGS IN PER CENT				REMARKS
	Total protein	Albumin	Globulin	Non-protein	
Before exchange.....	6.4	3.5	2.9	1.7	Slight shock
Immediately after....	2.1	1.3	0.8	1.3	
15 minutes after.....	3.2	2.2	1.0	1.6	
10 hours.....	3.9	2.7	1.2	1.6	Normal
2d day.....	4.5	2.6	1.9	1.9	

were injected into the right femoral vein. The duration of exchange was 14 minutes. Animal showed definite signs of intoxication after about an hour following the exchange, with some bloody feces after about 5½ hours. After 24 hours animal appeared to have recovered completely.

Experiment 96. (See table 5). 104 per cent exchange.

Dog 18-48. Young female bull dog. Weight 13.8 pounds. "Plasmapheresis," 99 per cent exchange with a duration of 14 minutes, done on October 4 (exper. 93, table 4). Showed definite signs of intoxication.

October 18, 1917. Under ether anesthesia 650 cc. blood were withdrawn from the left femoral artery. Simultaneously 650 cc. of blood corpuscle suspension were injected into the left femoral vein. *The duration of exchange was 2 minutes.* Immediately following the exchange there was a slight transient fall in temperature. There was no immediate alteration of the pulse; however after about an hour the pulse was of poor volume and the animal appeared decidedly dull. Bloody feces were noted at this time. After 24 hours the animal appeared to be in good condition.

Table 6 gives valuable data concerning the speed of exchange in its relation to shock and the curve of protein regeneration. In this experiment the blood volume exchange of 75 per cent was *completed in 2.5 minutes.* There was no shock and we may compare the previous exchanges done on this same animal (Sept. 12, 1917, exper. 84, 80 per

TABLE 6
75 per cent blood volume exchange; dog 18-35; experiment 94

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATO-CRIT RED CELL PER CENT	REMARKS
	Total protein	Albumin	Globulin	Non-protein			
Before exchange.....	5.4	3.8	1.6	1.9	0.21	40	
Immediately after.....	3.0	2.2	0.8	1.5	0.19	48	
15 minutes after.....	3.7	2.7	1.0	1.8	0.23	54	
3¼ hours.....	4.6	3.4	1.2	1.8	0.26	61	No shock
6¾ hours.....	4.7	3.5	1.2	1.8	0.30	44	
9½ hours.....	4.1	2.9	1.2	1.5			Normal
2d day.....	4.9	3.6	1.3	1.9	0.44		
3d day.....	4.4	3.1	1.3	2.8			
4th day.....	4.6	2.4	2.2	2.4			

cent exchange, shock very slight, time 7 minutes; October 3, 1917, exper. 92, 74 per cent exchange, no shock, time 14 minutes). The curve of serum protein depletion and regeneration is similar to other experiments. The small per cent exchange lowers the total protein to 3.0 per cent and the emergency increase is definite within 15 minutes, giving a rise to 3.7 per cent. It appears that the emergency reaction by which a considerable amount of serum protein is thrown into the blood stream, can be called out by a large or small exchange using this method.

Experiment 94. (See table 6). 75 per cent exchange.

Dog 18-35. Young female bull dog. Weight 16 pounds. "Plasmapheresis," 80 per cent exchange, done in 7 minutes on September 12, 1917, showing very

slight shock (exper. 84); another 14 per cent exchange done in 14 minutes on October 3, 1917, showing no shock (exper. 92). Blood volume on October 9, 1917 (by dye method) was 671 cc.

October 11, 1917. Under ether anesthesia 545 cc. blood were withdrawn from the right carotid artery. Simultaneously 545 cc. of blood corpuscle suspension were injected into the right external jugular vein. The duration of the exchange was $2\frac{1}{2}$ minutes. Animal showed a transient drop in temperature of 3 degrees immediately following the exchange. Animal showed little or no signs of intoxication.

Table 7 shows a remarkably prompt return to normal after a large exchange (109 per cent). The total proteins fell to a level of 50 per cent normal which is a normal reaction. We cannot explain the figures, which appear to show a peculiar reaction on the part of the albumin and globulin fractions. These peculiar reactions will appear at

TABLE 7

109 per cent blood volume exchange; dog 18-20; experiment 89

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATOCRIT RED CELL PER CENT	REMARKS
	Total protein	Albumin	Globulin	Non-protein			
Before exchange.....	5.0	3.6	1.4	1.9	0.35	50	
Immediately after...	2.5	0.9	1.6	1.4	0.12	60	
15 minutes after.....	3.0	1.5	1.5	1.6	0.11	73	
4½ hours.....	4.2	2.2	2.0	1.7	0.13	73	Very slight shock
2d day.....	5.1	2.9	2.2	2.1		41	Normal

times in spite of every care used in the method, but we are inclined to suspect technical errors as in part responsible. The return of the total protein to normal within 24 hours is unusual and would indicate an unusually large emergency reserve. The peculiar rise in red cell hematocrit will be found in this experiment and in a few subsequent experiments. That it appears in the 15-minute and 4-hour samples but not in the sample taken immediately after the exchange is very perplexing. There is no severe shock to account for any withdrawal of fluid from the blood. We have no convincing explanation to offer.

Experiment 89. (See table 7). 109 per cent exchange.

Dog 18-20. Young female bull dog. Weight 16.1 pounds.

September 20, 1917. Under ether anesthesia 800 cc. blood were withdrawn from the femoral artery. Simultaneously 800 cc. of blood corpuscle suspension were injected into the femoral vein. The duration of the exchange was $14\frac{1}{2}$

minutes. Animal showed little or no depression. The temperature fell 3 degrees, however, and the animal shivered considerably for 8 or 9 hours. In good condition after 24 hours.

Table 8 shows another typical experiment giving the usual curve of blood proteins following plasmapheresis of moderate amount (90 per cent). The *fibrin curve* is given in this experiment and we believe this illustrates the usual reaction on the part of this plasma globulin. The method used gives certain opportunities of error when small amounts of plasma are analyzed for fibrin. More work in this field has been completed by Mr. Foster in this laboratory and will soon be published. We do not wish to put too much emphasis on these figures.

TABLE 8
90 per cent blood volume exchange; dog 18-68; experiment 105

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	REMARKS
	Total protein	Albumin	Globulin	Non-protein		
Before exchange.....	5.5	4.0	1.5	2.2	0.42	
Immediately after.....	2.8	2.1	0.7	1.7	0.22	
15 minutes after.....	3.5	2.8	0.7	1.8	0.27	
2½ hours.....	4.6	3.3	1.3	1.8	0.30	Moderate shock
5 hours.....					0.19	
2d day.....	4.8	3.8	1.0	2.0	0.56	Normal
4th day.....	4.9	3.4	1.5	1.3	0.42	
6th day.....	5.4	3.7	1.7	2.1	0.49	

Experiment 105. (See table 8). 90 per cent exchange.

Dog 18-68. Young female bull dog. Weight 15.3 pounds.

November 21, 1917. Under ether anesthesia 623 cc. of blood were withdrawn from the right femoral artery. Simultaneously 623 cc. of blood corpuscle suspension were injected into the right femoral vein. The duration of the exchange was 9 minutes. The animal showed moderate depression and slight decrease in pulse volume for several hours.

Tables 9 and 10 give figures to show the low level of serum proteins which may be effected by very large blood volume exchanges (159 and 195 per cent). The usual normal dog will not tolerate such large exchanges without exhibiting profound and often fatal shock. These two dogs were unusually resistant to this experimental procedure and give us the opportunity to study the reaction following such large

exchanges uncomplicated by shock or notable hemolysis. The low level of total proteins is to be expected and one experiment (table 10) reaches the minimum figure for total protein (0.9 per cent). We have no observation in any of our experiments to show a lower level of protein in the blood stream. The protein regeneration is very rapid in the 15-minute period as well as in the following 24 hours, indicating considerable emergency reserve material.

TABLE 9

159 per cent blood volume exchange; dog 17-215; experiment 67

TIME	BLOOD SERUM READINGS IN PER CENT				REMARKS
	Total protein	Albumin	Globulin	Non-protein	
Before exchange.....	6.2	3.6	2.6	1.7	Slight hemolysis Slight hemolysis. Slight shock Normal
Immediately after....	1.3	0.3	1.0	1.6	
15 minutes after.....	2.2	1.2	1.0	1.5	
2d day.....	4.1	2.7	1.4	1.4	Normal
3d day.....	4.3	2.1	2.2	2.0	
4th day.....	5.2	2.9	2.3	1.7	

TABLE 10

195 per cent blood volume exchange; dog 17-232; experiment 70

TIME	BLOOD SERUM READINGS IN PER CENT				REMARKS
	Total protein	Albumin	Globulin	Non-protein	
Before exchange.....	6.3	3.7	1.6	1.9	Very slight shock Normal
Immediately after....	0.9	0.3	0.6	1.5	
15 minutes after.....	1.4	0.7	0.7	1.7	
2d day.....	4.3	2.3	2.0	1.5	
3d day.....	5.5	3.8	1.7	1.9	

Experiment 67. (See table 9). 159 per cent exchange.

Dog 17-215. Adult female fox terrier. Weight 15.25 pounds. Blood volume on July 19, 1917 (by dye method) was 858 cc.

July 31, 1917. Under ether anesthesia 1105 cc. of blood were withdrawn from the right femoral artery. Simultaneously 1105 cc. of blood corpuscle suspension were injected into the right femoral vein. The duration of the exchange was 10 minutes. There was slight decrease in force of pulse beat for about an hour. Animal showed little sign of depression thereafter.

Experiment 70. (See table 10). 195 per cent exchange.

Dog 17-232. Young female coach dog. Weight 13.5 pounds. Blood volume on July 19, 1917 (by dye method) was 714 cc.

August 3, 1917. Under ether anesthesia 1200 cc. blood were withdrawn from the right femoral artery. Simultaneously 1200 cc. of blood corpuscle suspension were injected into the right femoral vein. The duration of the exchange was 10 minutes. The pulse pressure was poor for about an hour following the exchange.

TABLE 11

PERCENTAGE EXCHANGE	EXPERIMENT NUMBER	TOTAL PROTEIN				TOTAL DROP IN PROTEIN	PER CENT PROTEIN REGAINED		CLINICAL SHOCK	HEMATOCRIT (PER CENT BLOOD CELLS)			
		Before exchange	At end of exchange	15 minutes after exchange	Second day		In 15 minutes	In 24 hours		Before exchange	At end of exchange	15 minutes after exchange	Second day
67	60	5.3	2.8	3.9		2.5	1.1		0				
74	92	5.5	3.7	3.8		1.8	0.1		0	50	58	58	
74	87	6.2	3.3	3.9		2.9	0.6		+++	58	58	55	
75	94	5.4	3.0	3.7	4.9	2.4	0.7	1.9	0	40	54	54	
75	90	5.3	2.9	3.4	4.3	2.4	0.5	1.4	++	48	54	55	38
80	84	5.6	3.1	3.1	6.6	2.5	0.0	3.5	+	50	63	58	33
80	82	5.6	2.4		4.4	3.2		2.0	0		50		44
84	74	4.9	2.6			2.3			*				
89	101	5.5	2.1	2.4		3.4	0.2		+++	60	41	53	
90	105	5.5	2.8	3.5	4.8	2.7	0.7	2.0	++	43	49	52	
91	103	6.2	3.2	3.8	4.3	3.0	0.6	1.1	++	49	49	56	35
94	62	4.9	1.8	2.8	4.8	3.1	1.0	3.0	++				
96	61	5.5	2.7	3.6		2.8	0.9		0				
98	64	6.3	3.0	2.8		3.3	-0.2		+				
99	81	4.5	1.2	2.5	4.1	3.3	1.3	2.9	++				
99	93	4.5	2.0	3.3	4.0	2.5	1.3	2.0	+	58	58	58	42
100	100	5.6	3.0	2.9	4.6	2.6	-0.1	1.6	+	46	62	64	36
Averages		5.4	2.7	3.3	4.8	2.7	0.6	2.1		50	55	56	38

Shock readings: + means slight shock; ++ means moderate to severe shock; +++ means lethal shock.

* Death from overdose of ether.

A summary of certain factors in many plasma depletion experiments will be found in tables 11 and 12 and the *average figures* give much interesting information. The averages of the experiments which show 100 per cent or less of blood volume exchange (table 11) show an identical emergency increase in the blood serum proteins. The two tables are practically in accord and we note that the average replacement of serum protein during the 15 minutes following the plasmapheresis

amounts to 0.5 to 0.7 per cent protein—which is an increase of 10 to 14 per cent of the total proteins. The increase during the 24 hours following the plasma depletion is considerable and amounts to 2.0 per cent protein which is an increase of 40 per cent total protein, figuring 5.0 protein per cent as the normal for a healthy dog.

Further analysis of the blood cell hematocrit figures is of interest. It is unfortunate that we did not obtain hematocrit readings in all our experiments. It is clear that the normal hematocrit before the ex-

TABLE 12

PERCENTAGE EXCHANGE	EXPERIMENT NUMBER	TOTAL PROTEIN				TOTAL DROP IN PROTEIN	PER CENT PROTEIN REGAINED		CLINICAL SHOCK	HEMATOCRIT (PER CENT BLOOD CELLS)			
		Before exchange	At end of exchange	15 minutes after exchange	Second day		In 15 minutes	In 24 hours		Before exchange	At end of exchange	15 minutes after exchange	Second day
102	80	5.6	2.6	2.8		3.0	0.2		+++				
104	96	6.4	2.1	3.2	4.5	4.3	1.1	2.4	+	52	66		38
108	98	6.3	2.2	2.7		4.1	0.5		+++	33	44	53	
109	89	5.0	2.5	3.0	5.1	2.5	0.5	2.6	+	50	60	73	41
110	83	5.8	2.3	2.8	4.2	3.5	0.5	1.9	+				46
118	63	7.1	4.2		4.8	2.9		-0.1	+				
122	104	5.7	1.9	2.3		3.8	0.4		+++				
131	66	5.7	1.0	3.1	4.0	4.1	1.5	2.4	++	47	35	38	30
141	68	5.5	2.6	2.5	3.9	2.9	-0.1	1.3	0				
159	67	6.2	1.3	2.2	4.1	4.9	0.9	2.8	+				
170	69	5.6	2.0	2.9	4.2	3.6	0.9	2.2	++				
191	77	5.9	0.9	1.2		5.0	0.3		+++				
195	70	5.3	0.9	1.4	4.3	4.4	0.5	3.4	0				
Averages		5.8	2.0	2.5	4.3	3.8	0.6	2.1		46	51	55	39

Shock readings: + means slight shock; ++ means moderate to severe shock; +++ means lethal shock.

periment is approximately 50, which is an indication that healthy dogs were used. There is a slight increase in the hematocrit figures at the end of the blood exchange but only to 55 per cent. This assures us that a suitable number of red cells was introduced in the red cell Locke's solution mixture which replaced the blood. There is a trifling increase in the average hematocrit figures for the 15-minute sample but only to 56 per cent, which may indicate a very slight blood concentration due to loss of Locke's solution from the circulation.

There is a distinct fall in hematocrit during the 24 hours following the plasmapheresis—an average of 7 to 12 per cent below the initial figure. This probably indicates a true loss of red cells as we must recall the fact that these red corpuscles which are introduced have

TABLE 13

PERCENTAGE EXCHANGE	EXPERIMENT NUMBER	FIBRIN IN PER CENT				TOTAL DROP IN FIBRIN	PER CENT FIBRIN REGAINED		CLINICAL SHOCK	HEMATOCRIT (PER CENT BLOOD CELLS)			
		Before exchange	At end of exchange	15 minutes after	Second day		In 15 minutes	In 24 hours		Before exchange	At end of exchange	15 minutes after exchange	Second day
74	92	0.21	0.16	0.15	0.28	0.05	-0.01	0.12	0	50	58	58	
74	87	0.18	0.08	0.15		0.10	0.07		+++	58	58	55	
75	94	0.21	0.19	0.23	0.44	0.02	0.03	0.25	0	40	48	54	
75	90	0.25	0.15	0.20	0.35	0.10	0.05	0.20	++	48	54	55	38
80	84	0.17	0.06	0.03	0.40	0.11	-0.03	0.34	+	50	63	58	33
80	82	0.17	0.08	0.13	0.33	0.09	0.05	0.25	0		50		44
84	74	0.36	0.15			0.21			*				
89	101	0.24	0.11	0.11		0.13	0.00		+++	60	41	53	
90	105	0.42	0.22	0.27	0.56	0.20	0.05	0.34	++	43	49	52	
91	103	0.42	0.19	0.21	0.48	0.23	0.02	0.29	++	49	49	56	35
99	93	0.26	0.16	0.15	0.30	0.10	-0.01	0.14	+	58	58	58	42
99	81	0.32		0.19					++				
100	100	0.25	0.16	0.16	0.45	0.09	0.00	0.29	+	46	62	64	36
102	80	0.42		0.26					+++				
104	96	0.50	0.13	0.14	0.40	0.37	0.01	0.27	+	52	66		38
108	98	0.25	0.12	0.11		0.13	-0.01		+++	33	44	53	
109	89	0.35	0.12	0.11		0.23	-0.01		+	50	60	73	41
110	83	0.75	0.18	0.30	0.44	0.57	0.12	0.26	+				46
122	104	0.50	0.13	0.14	0.40	0.37	0.01	0.27	+++	52	66		38
191	77	0.44	0.13	0.10		0.31	-0.03		+++				
Averages..		0.33	0.14	0.17	0.40	0.19	0.02	0.25		49	55	57	39

Shock readings: + means slight shock; ++ means moderate to severe shock; +++ means lethal shock.

* Death from overdose of ether.

been submitted to considerable manipulation in the necessary washing previous to the injection. Dogs' corpuscles too are notoriously fragile. We may assume for the present at any rate that many of the red cells which were introduced had been seriously injured and went to

pieces in the blood stream during the 24 hours following the blood exchange.

We can review the *fibrin analyses* in table 13 and at once a decided difference appears when we compare the serum protein curve with that of the plasma globulin, fibrinogen. The exchange of blood reduces the fibrin content to about the same level—that is, we can wash out the same percentage of fibrinogen by the usual plasmapheresis as we do in the case of the serum proteins. The fibrin content is reduced to a little less than one-half normal—from 0.33 to 0.14. During the 15 minutes following the plasma depletion there is no emergency reaction on the part of the fibrin as is so constant for the serum proteins. During the next 24 hours the fibrin is restored completely to normal. This may mean that there is no emergency reserve of the fibrin as it can be produced so rapidly in the body in any emergency. We know of many other facts which point to *complete dissociation* of fibrin and other blood proteins as to production and repair and general usefulness in the body economy.

DISCUSSION

A theoretical consideration of the factors involved in this protein replacement is difficult at this time. It may be claimed that this increase represents, in part at least, not a true increase in the quantity of circulating serum protein, but is the result of the escape from the circulation of fluid poor in protein material. However, if any considerable escape of fluid from the circulation were to occur, one would expect to note a rise in the percentage of red cells in the circulating medium. That no sufficient change in the cell-plasma ratio does occur can be seen from the hematocrit figures given in tables 11 and 12. On the other hand, it may be that the figures represent an actual influx of protein into the circulating medium. Such an influx could conceivably come from some tissue or organ which serves as a storehouse for this type of protein material. Seitz (7) thinks that the liver acts as such a storehouse. Earlier work in this laboratory by Kerr, Hurwitz and Whipple (1) shows a lack of reserve production of serum proteins after plasmapheresis in the Eck fistula dog. This indicates that liver insufficiency may impair the normal emergency reproduction of blood proteins.

Of particular interest is the rather remarkable increase in the 15 minutes immediately following the end of the experimental depletion.

This, if blood volume changes be excluded, appears to be truly a throwing in of ready-formed materials.

While the depletion curve of the fibrin fraction of the plasma proteins brought about by our experimental procedure compares closely with the curve of depletion of the serum proteins, still a distinctly different type of curve of fibrin repletion is revealed. A fairly typical experiment is presented in table 8. This point may also be studied by an examination of the results given in table 13. In these tables it may be seen that the rapid rise immediately following the procedure of depletion which is typical of the serum proteins is absent or at least negligible in the case of fibrin. However, the body seems to be able to supply large amounts of this protein in a space of 24 hours, for as the summary in table 13 shows, the fibrin on the day following the exchange is already as high as the original figure, or, as occurs in some cases, even higher. When such an over-production does occur the level usually returns to normal in one to two days.

This lack of correspondence between the regeneration figures for serum proteins and for fibrin protein in the period of initial regeneration, we believe furnishes additional evidence against a theory which would account for all changes in protein concentration in this period by the loss from the circulation of fluids poor in protein. For, in such a case, the concentration of the proteins might be expected to occur to practically the same degree in each. That this does not occur tends to strengthen the evidence given by the hematocrit figures.

It may be pointed out that the curve of serum protein regeneration is very different for this type of experiment when compared to the experiments of Kerr, Hurwitz and Whipple. We believe that these differences are to be explained wholly by the differences in the experimental depletion of the serum protein. Kerr, Hurwitz and Whipple used *interval depletions* of smaller amounts but repeated many times during a single day. In this manner they undoubtedly removed much of the large emergency reserve which is so conspicuous in the 24-hour regeneration in the experiments tabulated above. Therefore Kerr, Hurwitz and Whipple observed a curve of protein regeneration which was much more prolonged before a return to normal was observed. These experiments supplement the earlier ones and strengthen their conclusions.

SUMMARY

A rapid depletion of serum proteins is brought about in these experiments by the introduction of normal red blood cells suspended in a modified Locke's solution, care being taken to keep equivalent the volume of blood removed from the artery and the volume of red blood cell suspension simultaneously injected into the vein.

The serum protein depletion is roughly proportional to the size of this exchange and it is noteworthy that the rapid depletion of the total serum proteins can rarely be carried below 1.0 per cent without causing a fatal reaction.

An increase in serum protein concentration (serum protein replacement) begins immediately following the exchange or plasmapheresis. The increase is very rapid during the first 15 minutes following the exchange. The increase in serum proteins is more gradual thereafter during the first 24 hours and still more sluggish during the next few days. The normal level may be reached in 2 to 7 days.

The rapid replacement of serum proteins during the first 15 minutes following the exchange indicates some reserve supply of this material perhaps held in the body cells. The emergency supply is evidently small and the production of other similar material is difficult and requires time.

The blood fibrin reacts in a different fashion. The same initial fall is not followed by a rapid rise in the first 15 minutes. The recovery however is complete within 24 hours and probably earlier than this. Fibrin is a very labile protein as compared with the serum albumin and globulin.

Blood volume fluctuations are probably very little concerned in these experimental results. The red blood cell hematocrit ratio shows but little change during the period of initial reaction.

BIBLIOGRAPHY

- (1) KERR, HURWITZ AND WHIPPLE: *This Journal*, 1918, xlvii, 356, 370, 379.
- (2) ABEL, ROWNTREE AND TURNER: *Journ. Pharm. Exper. Therap.*, 1914, v, 625.
- (3) MORAWITZ: *Beitr. z. chem. Physiol. u. Pathol.*, 1906, vii, 153.
- (4) HOOPER, SMITH, BELT AND WHIPPLE: *This Journal*, 1920, li, 205.
- (5) ROBERTSON: *Journ. Biol. Chem.*, 1915, xxii, 233.
- (6) CULLEN AND VAN SLYKE: *Proc. Soc. Exper. Biol. and Med.*, 1916, xiii, 197.
- (7) SEITZ: *Arch. gesamt. Physiol.*, 1906, cxi, 309.

II. SHOCK AS A MANIFESTATION OF TISSUE INJURY FOLLOWING RAPID PLASMA PROTEIN DEPLETION

THE STABILIZING VALUE OF PLASMA PROTEINS

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In the preceding communication we have established the curve of serum protein regeneration following a single rapid replacement of whole blood with a red cell Locke's solution mixture. This plasma depletion (plasmapheresis) washes out more or less of the blood proteins and lowers the concentration of the blood proteins in the circulating blood. In the preceding article we have submitted many experiments which are associated with little or no "shock," using this term in the familiar clinical sense. In this paper we wish to discuss more particularly those cases which are associated with severe or lethal shock. A number of such experiments are given in detail below. This intoxication associated with plasmapheresis has been noted by the earlier workers: Morawitz (1), Abel, Rowntree and Turner (2) and Kerr, Hurwitz and Whipple (3). A variety of explanations has been given.

The *physiological value of the serum proteins* is admittedly little understood and we believe our experiments throw some light on this point. Published work from this laboratory (3) indicates that the serum proteins cannot be concerned with the nutrition of the body cells and the constant exchange between food protein and body protein. The experiments outlined below suggest rather strongly that one important function of these proteins is their "stabilizing value."

The *stabilizing value* of the blood serum proteins is brought out with especial emphasis by two experiments (tables 19 and 20). The dog is bled large amounts from the femoral artery while simultaneously equal amounts of a washed red cell, dialyzed serum mixture are injected into the femoral vein. No shock followed an exchange of

large size which would surely have been fatal if the dialyzed serum had been replaced by Locke's solution as in the standard plasmapheresis. During the dialysis of the serum it underwent considerable dilution while the dialyzable substances were being removed, but this dilute dialyzed serum was still able to protect the body cells against the shock which develops if the blood proteins are too much diluted as in the routine plasma depletion. We believe that this furnishes the last bit of evidence to show that the blood serum proteins make up an essential part of the environmental complex of the body cells. Too great a dilution of these substances invariably results in profound injury of certain cells and a reaction identical with "clinical shock."

When these protein substances are suddenly washed out of the blood serum there is a certain amount of similar material thrown in as an emergency reserve. If the depletion is too severe the body cells are injured by the very persistence of this abnormal condition and the condition of "shock" supervenes. Further it is evident that certain body cells are more sensitive than others to changes in the serum protein content—for example, liver cells. That these facts have some significance in relation to the general problem of clinical shock is at once evident.

Other experiments (3) already cited give proof that the simple plasma depletion with more or less clinical shock is associated with a certain amount of cell injury, as shown by the rise in urinary nitrogen in the two days following the exchange. There is neither gross nor histological evidence of cell necrosis, but this increase in nitrogen must come from body protein. This is further evidence for actual *cell injury as an essential part of the clinical complex named "shock."*

It may be noted also that when once the clinical picture of "shock" is established in these experiments we have been unable to save the animal by any of the familiar clinical measures, even by infusion of whole blood. The *essential injury* in these experiments is *cell protoplasm injury* induced by a sudden change in the colloidal solution which forms the normal environment of these cells. This may be a new type of cell injury but it may help us to understand the more complex cell injury which is probably responsible for "surgical shock."

EXPERIMENTAL OBSERVATIONS

The various experimental methods have been described in detail in the preceding communication. To save repetition we may refer to some of the experiments detailed in the first paper of this series. The experiments given below are only types which illustrate a characteristic reaction and usually represent groups of similar experiments.

The first two tabulated experiments (tables 14 and 15) illustrate the reaction which was so common in the experiments of paper I of this series. In addition these two experiments done on the same dog at an interval of three weeks show that this procedure (plasmapheresis) does not sensitize a dog to any subsequent repetition of this procedure. This shock so exactly resembles the anaphylactic shock in dogs that it seemed necessary to exclude this possibility. Other experiments giving the same negative results need not be instanced.

TABLE 14

80 per cent blood volume exchange; very slight shock; dog 18-35; experiment 84

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATO-CRIT RED CELL PER CENT
	Total protein	Albumin	Globulin	Non-protein		
Before exchange.....	5.6	4.6	1.0	2.4	0.17	50
Immediately after.....	3.1	2.5	0.6	2.8	0.06	63
15 minutes after.....	3.1	2.9	0.2	2.6	0.03	58
4 hours.....	4.1	3.3	0.8	2.5	0.06	58
8 hours.....	6.1	4.9	1.2	2.5	0.16	45
2nd day.....	6.6	5.5	1.1	2.7	0.40	33

The curve of protein regeneration during the eight hours following this plasma depletion is beautifully shown in both experiments. The emergency reserve was sufficient to replace all the serum proteins removed (table 14) but it is noted that the total drop in serum protein was but 2.5 per cent total protein.

Experiment 84. (See table 14). 80 per cent exchange.

Dog 18-35. Female bull pup. Weight 15 pounds. Appears to be in excellent condition.

September 12. Under ether anesthesia 545 cc. of blood were withdrawn from the right femoral artery. Simultaneously and at the same rate 545 cc. of Locke's corpuscle suspension were injected into the right femoral vein. The exchange was effected in 7 minutes. There was a fall in rectal temperature of about 1°C. following the exchange. No definite sign of intoxication was noted except for a slight amount of vomiting 2 hours following the exchange. The animal appeared to be in good condition on the 2nd day.

Experiment 92. (See table 15). 74 per cent exchange.

Dog 18-85. Female bull pup. Weight 16.25 pounds. On September 12 an 80 per cent exchange was effected in 7 minutes without any decided signs of intoxication (see table 14).

October 3. Dog seems to be in excellent condition. Under ether anesthesia 545 cc. of blood were withdrawn from the left femoral artery. Simultaneously and at the same rate 545 cc. of Locke's corpuscle suspension were injected into the left femoral vein. The exchange was effected in 14 minutes. There was practically no alteration in rectal temperature and at no time were there any signs of intoxication.

Experiment 61. No clinical shock. 96 per cent exchange.

Dog 18-7. Female terrier pup. Weight 11 pounds. Estimated blood volume (by dye method) 530 cc.

July 19. Under ether anesthesia 480 cc. of blood were withdrawn from the right femoral artery. Simultaneously and at the same rate 480 cc. of Locke-corpuscle suspension were injected into the right femoral vein. The duration of the exchange was 12 minutes. There was practically no disturbance in rectal temper-

TABLE 15

74 per cent blood volume exchange; no clinical shock; dog 18-85; experiment 92

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATO-CRIT RED CELL PER CENT
	Total protein	Albumin	Globulin	Non-protein		
Before exchange.....	5.5	4.0	1.5	1.8	0.21	50
Immediately after.....	3.7	2.7	1.0	1.7	0.16	58
15 minutes after.....	3.8	2.9	0.9	1.8	0.15	58
2½ hours.....	4.3	3.4	0.9	1.8	0.18	48
5½ hours.....	4.6	3.5	1.1	1.8	0.21	48
2nd day.....					0.28	

ature. There was a slight amount of drowsiness for several hours. Otherwise no disturbance was noted. The total serum proteins fell from the initial value of 5.5 per cent at the beginning of the exchange to a level of 2.7 per cent at the end of the exchange. Fifteen minutes later a value 3.6 per cent was found. No samples were taken subsequently. At no time was there any decided alteration in the albumin-globulin ratio.

The next group of experiments illustrates the fatal shock which may develop following an exchange of blood equal to 100 per cent blood volume or more. From these and other experiments it is obvious that the body can supply an emergency reserve of serum proteins even during the period of profound shock which precedes death (2 to 5 hours). Moreover the ratio of albumin and globulin is not especially disturbed as is so frequently seen in severe intoxication due to bacterial invasion.

The clinical and anatomical pictures described in this condition of shock following plasma depletion are very constant and resemble in the dog the reaction observed in fatal anaphylaxis. The fall in blood pressure may be delayed several minutes—sometimes 30 minutes after completion of the exchange—but the fall in temperature is prompt. At times there may be a subsequent rise in temperature even in fatal intoxication, but often the loss of temperature control is complete and rectal temperatures of 30°C. may be recorded. Gastro-intestinal disturbance is the rule. Vomiting and diarrhea are seen early, sometimes within 30 minutes, and persist. This watery, blood-tinged diarrhea is common in fatal cases. Mucus may be very abundant in certain cases, even occasionally when recovery takes place following a severe intoxication. The dull lethargic appearance with clinical prostration is very typical of this type of shock. This picture corresponds closely with the surgical condition of "shock" associated with intoxication (for example, intestinal obstruction) or hemorrhages or prolonged operative manipulation.

The *autopsy findings* also are very uniform. For these the description of a single case will suffice. Blood removed from the heart at autopsy or from the veins at intervals before death may show delayed coagulation but this is not uniform. The fibrin content is low because this plasma protein like the serum proteins has been washed out by the exchange. The liver, spleen and kidneys show engorgement, usually most marked in spleen and liver. The thorax, heart and lungs are negative. The stomach may be pale or slightly injected. The entire small intestine shows congestion of its mucosa often more marked in the upper tract. The mucosa may be velvety, purplish red and coated with thick creamy mucous. All degrees of congestion are found. The lumen contains a thin, watery, blood-tinged fluid in which more or less mucus is present. The colon shows the same material and a mottled congested mucosa.

It will be noted that this picture of shock is almost identical with that produced by large doses of adrenalin, clamping of aorta or vena cava and trauma of the intestines, recently studied and described by Erlanger (5).

Experiment 98. (See table 16). 108 per cent exchange.

Dog 18-20. Female bull-terrier pup. Weight 19.4 pounds. On September 20 an exchange of 109 per cent in 14½ minutes produced moderately severe shock.

October 31. Under ether anesthesia 950 cc. of blood were withdrawn from the left femoral artery. Simultaneously and at about the same rate 1000 cc. of Locke's corpuscle suspension were injected into the left femoral vein. The duration of

the exchange was $12\frac{1}{2}$ minutes. The rectal temperature fell about $1^{\circ}\text{C}.$ during the exchange. Subsequently there was a fall of 1° more, when death occurred. The arterial tension was fairly good at the end of the exchange but became quite poor in the course of the next 15 minutes. It remained poor until death. Deep respiration developed in the course of the first hour following the exchange. No marked signs of depression or loss of power of attention appeared for about 2 hours after the exchange. The condition then became rapidly worse and death occurred 1 hour later.

Autopsy shows swollen congested spleen. The liver is deep red, the lobulation is obscure. The mucosa of the entire intestinal tract is congested. There is a considerable excess of mucus. The other organs are negative. Blood drawn from the heart at time of autopsy when placed in a test tube clots in 25 minutes; that which is left in contact with the tissues clots in 10 minutes. The clot formed is quite flabby.

Experiment 101. (See table 17). 89 per cent exchange.

Dog 18-5. Young male terrier. Weight 18.5 pounds. On July 18 an exchange of 67 per cent produced a very mild grade of shock.

TABLE 16

108 per cent blood volume exchange; fatal shock; dog 18-20; experiment 98

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATO-CRIT RED CELL PER CENT	REMARKS
	Total protein	Al-bumin	Globulin	Non-protein			
Before exchange	6.3	4.4	1.9	2.1	0.25	33	
Immediately after	2.2	1.3	0.9	1.4	0.12	44	
15 minutes after	2.7	1.7	1.0	1.6	0.11	53	
2 hours	3.6	2.5	1.1	2.1	0.12	42	Death

November 8. Under ether anesthesia 750 cc. of blood were withdrawn from the left femoral artery. Simultaneously 750 cc. of Locke's corpuscle suspension were injected into the left femoral vein. The duration of the exchange was 11 minutes. The rectal temperature fell $2^{\circ}\text{C}.$ as a result of the exchange but returned subsequently to the original level of slightly above 40° . The *arterial tension* was good at the end of the exchange but became poor within the course of 30 minutes. Definite signs of general depression or "shock" appeared within an hour following the end of the exchange. The power of attention was completely lost 2 hours later. Death occurred 5 hours after the exchange.

Autopsy: The thymus is somewhat larger than normal. Otherwise the thoracic organs are negative. The spleen is moderately enlarged and congested. The liver is negative except for pronounced indistinctness of lobulation. The mucosa of the duodenum is slightly reddened, but no excess of mucus is found in the lumen.

Experiment 76. Fatal shock. 178 per cent exchange.

Dog 18-7. Female terrier pup. Weight 10.5 pounds.

A 96 per cent exchange was carried out on July 19 (see exper. 61) with practically no sign of shock.

On July 26 an exchange of 118 per cent was effected with very slight reaction. Immediately following this second exchange an injection of phosphorus was given. No definite injury was noted (see exper. 65, table 24).

August 16. Animal appeared to be in excellent condition. Under ether anesthesia 850 cc. of blood were withdrawn from the right carotid artery. Simultaneously and at about the same rate 900 cc. of Locke's corpuscle suspension were injected into the right external jugular vein. The duration of the exchange was 7 minutes. Within a few minutes definite signs of shock appeared. The pulse rapidly diminished in volume, the respiration became irregular and the rectal temperature fell steadily from the original of 38.5°C. to 36.1° at the time of death, 1½ hours following the exchange.

Autopsy: The thoracic organs are negative. The spleen, liver and kidneys show moderate congestion. The upper part of the small intestines shows marked thickening and congestion of the mucosa. Thin bloody fluid is present in considerable quantities within the lumen of the intestines. The mucosa of the large intestine is slightly congested. The pancreas is decidedly swollen by interlobular edema. There is a considerable amount of hemolysis.

TABLE 17

89 per cent blood volume exchange; fatal shock; Dog 18-5; experiment 101

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATOCRIT RED CELL PER CENT	REMARKS
	Total protein	Albumin	Globulin	Non-protein			
Before exchange.....	5.5	4.4	1.1	2.2	0.24	60	
Immediately after.....	2.1	1.7	0.4	1.7	0.11	41	
15 minutes after.....	2.4	1.8	0.6	2.2	0.11	53	
3 hours.....					0.13	54	
5 hours.....	3.6	2.6	1.0	2.2	0.21	56	Death

The refractometric estimation of serum proteins was not carried out. Nitrogen estimation by the Kjeldahl method showed that the total plasma proteins decreased as a result of the exchange from 4.9 per cent to 2.0 per cent. The fibrin content of the plasma fell from a level of 0.44 per cent to a level of 0.19 per cent as a result of the exchange. The value at the end of 15 minutes was 0.15 per cent, and 0.24 per cent at autopsy.

From a perusal of many experiments in this paper it is evident that there are wide individual variations in the susceptibility of different dogs to the plasma depletion. But each individual dog will usually react with considerable uniformity to a repeated plasmapheresis of unit volume if sufficient time is allowed between experiments for complete recovery. This is noted in tables 14 and 15 which give data from two experiments performed on the same animal at 3 weeks interval. If the second or succeeding exchanges are larger in amount

we may expect to record increasing degrees of intoxication and finally severe or fatal shock. This fact is illustrated by the preceding experiment (no. 76) in which two previous plasma depletions had no ill effects. The first one was an exchange of only 96 per cent with no signs of intoxication. The second exchange was slightly larger (118 per cent) and caused a slight intoxication. The final exchange of 178 per cent caused a prompt and fatal intoxication with the characteristic post-mortem findings described in fatal shock.

The substitution of serum for Locke's solution in plasma depletion (fibrinphoresis) Experiment 323. (See table 18). 144 per cent exchange.

Dog 19-74. Adult female mongrel terrier. Weight 16 pounds.

August 2. Under ether anesthesia 1050 cc. of blood were withdrawn from the right femoral artery. Simultaneously and at the same rate 1100 cc. of a serum corpuscle mixture were injected into the right femoral vein. This serum corpuscle mixture consisted of 550 cc. of packed dog corpuscles washed twice with sterile

TABLE 18

144 per cent blood volume exchange; substitution of serum for Locke's solution in plasma depletion; no clinical shock; dog 19-74; experiment 323

TIME	TOTAL SERUM PROTEINS PER CENT	HEMATOCRIT RED CELL PER CENT	FIBRIN IN PER CENT
Before exchange.....	6.3	58	0.60
Immediately after.....	5.4	49	0.08
15 minutes after.....	5.8	54	•
3 hours.....		50	0.30
24 hours.....	5.3	45	0.50

calcium-free Locke's solution in the customary way, to which was added an equal amount of serum. The serum for this purpose was obtained by drawing into large centrifuge tubes blood from normal dogs. After the process of clotting was completed the tubes were centrifugalized and the supernatant serum withdrawn. The duration of the exchange was 6 minutes. Ether anesthesia lasted 1 hour. The rectal temperature fell $2\frac{1}{2}^{\circ}\text{C}$. as a result of the procedure but returned to the original level within the space of about 2 hours. The arterial tension was good at the end of the exchange. There was at no time any definite impairment in the quality of the pulse. The animal showed no signs of shock.

August 3. Dog in excellent condition.

The substitution of serum for Locke's solution in the plasma depletion (fibrinphoresis)

Experiment 324. 176 per cent exchange. Female bull-terrier pup (3 months old). Weight 15 pounds.

August 5. Under ether anesthesia 1200 cc. of blood were withdrawn from the right femoral artery. Simultaneously and at the same rate 1275 cc. of serum corpuscle suspension made up as described in experiment 323 were injected into the right femoral vein. The duration of the exchange was 15 minutes. Ether

anesthesia lasted 40 minutes. The rectal temperature was depressed about 3°C. for a period of about 2 hours. The animal remained quiet for a period of 1 hour following the exchange, the power of attention being, however, good at all times. At the end of this time the animal was in excellent condition. The hematocrit values fluctuated but slightly as a result of the experimental exchange. The fibrin content of the plasma fell from its normal level of 0.43 to 0.30 per cent 3 hours after the exchange. The reading after 24 hours was 0.65 per cent.

The two preceding experiments (table 18, expts. 323 and 324) bring out several important facts. The experimental manipulation of the red cells and the actual exchange of one mass of red cells for another are not responsible for the intoxication. In these two experiments we employed washed red cells from normal dogs prepared exactly as described for other experiments. These cells were suspended *not in Locke's solution* but in the proper amount of *fresh normal dog serum*. These large exchanges then did not wash out any serum proteins but did remove much of the fibrin. These experiments serve as good controls of the operative procedures. These large exchanges gave no evidence of any resultant intoxication. The last one especially (exper. 324) was a particularly large exchange (176 per cent) and done upon a young dog. Our experience shows that young animals as compared with adults are more sensitive to the shock of plasma depletion.

•
One hundred and fifty per cent exchange using washed corpuscles suspended in dialyzed serum

Experiment 327. (See table 19).

One thousand cubic centimeters of blood were drawn from normal dogs, poured immediately into large centrifuge tubes and allowed to clot. The clot formed in each tube was freed from the side of the tube and the tube centrifugalized. The supernatant serum was removed. Three hundred cubic centimeters of this serum were then placed in 15 celloidin sacs which were then immersed in 5,000 cc. of Locke's solution containing no calcium or glucose and made about 10 per cent more concentrated than normal in order that the increased osmotic pressure might in part overcome the tendency of the serum proteins to dilute themselves by attraction of water from the surrounding fluid. After dialysis had proceeded for 4 hours the modified Locke's solution was replaced by 10,000 cc. more of fresh solution of the same constitution. Dialysis was then continued for 11 hours, at the end of which time the serum contained in the celloidin sacs had increased from 300 cc. to 450 cc. To 400 cc. of the dialyzed serum 600 cc. of dog corpuscles twice washed with calcium-free Locke's solution in the ordinary way were added. The mixture was strained and heated to 38°C.

Under ether anesthesia the entire 1000 cc. of the serum corpuscle mixture were injected into the right femoral vein of a normal short-haired bull pup weighing

12.5 pounds. Simultaneously and with moderate fluctuations in the rate of flow, 850 cc. of blood were withdrawn from the right femoral artery. Thirty-five minutes were consumed in effecting the exchange. The animal showed but little alteration in body temperature as a result of the exchange. Consciousness returned shortly after the discontinuance of the anesthetic. The animal was somewhat quiet for a period of about 45 minutes. Subsequently he was bright and apparently in very good condition.

One hundred and ninety-nine per cent exchange using washed corpuscles suspended in dialyzed serum

Experiment 329. (See table 20). Nine hundred cubic centimeters of blood were drawn from normal dogs, poured immediately into large centrifuge tubes and allowed to clot. The clot formed in each tube was freed from the side of the tube and the tube centrifugalized. The supernatant serum was removed. Three hundred and fifty cubic centimeters of this serum were then placed in 18 cel-

TABLE 19

150 per cent blood volume exchange using washed corpuscles suspended in dialyzed serum; experiment 327

SAMPLE	BLOOD SERUM READINGS IN PER CENT			
	Total protein	Albumin	Globulin	Non-protein
Of serum of perfusate:				
Before dialysis.....	6.2	3.8	2.4	1.7
After dialysis.....	3.3	2.5	0.8	1.2
Of dog perfused:				
Before exchange.....	5.7	3.0	2.7	1.9
Immediately after.....	3.8	2.1	1.7	1.9
4 hours after.....	4.5	2.3	2.2	2.0

loidin sacs which were then immersed in 4000 cc. of Locke's solution containing no calcium or glucose, and made about 10 per cent more concentrated than normal. After dialysis had proceeded for 5 hours the modified Locke's solution was replaced by 9000 cc. of fresh modified Locke's solution. Dialysis was then continued for 10 hours, at the end of which time the serum contained within the celloidin sacs had increased from 350 cc. to 450 cc. To 400 cc. of the dialyzed serum 600 cc. of dog corpuscles twice washed in calcium-free Locke's solution in the ordinary manner, were added. The mixture was strained and warmed to 38°C.

A normal short-haired black female mongrel terrier (no. 20-62), weighing 10.2 pounds, was anesthetized with ether and the entire corpuscle suspension was injected into the right femoral vein. Simultaneously and at the same rate 925 cc. of blood were withdrawn from the right femoral artery. The exchange was effected in 10 minutes. The temperature fell to 34.7°C. immediately following the exchange but under the influence of the heat-pad returned to 37.5°C. within a space of about 1½ hour. The animal regained consciousness within about 30

minutes following the exchange and was rather quiet for another 30 minutes, but thereafter appeared to be quite normal. The pulse was at no time markedly depressed.

The two experiments, tables 19 and 20, confirm the two preceding experiments (table 18) using fresh dog's serum. Suspension of washed red blood cells in fresh dialyzed dog serum (tables 19 and 20) gives a mixture which can be used in almost unlimited amounts to exchange with whole blood by the method adopted. This exchange is associated with no clinical shock. There is a slight lowering in the concentration of blood serum protein and of course the fibrinogen is almost completely washed out of the blood. This fibrinogen, however, can be reproduced rapidly and gives no clinical reaction as its normal content is reestablished in the blood in a few hours.

TABLE 20

199 per cent blood volume exchange using washed corpuscles suspended in dialyzed serum; experiment 329

SAMPLE	BLOOD SERUM READINGS IN PER CENT				HEMATO-CRIT RED CELL PER CENT	UREA NITROGEN PER 100 cc.	NON-PROTEIN NITROGEN PER 100 cc.
	Total protein	Albumin	Globulin	Non-protein		mgm.	mgm.
Serum of perfusate:							
Before dialysis.....	6.7	4.0	2.7	1.5		20	40
After dialysis.....	5.1	3.5	1.6	1.0		2	16
Of dog perfused:							
Before exchange.....	5.9	4.3	1.6	1.6	45.1		
Immediately after....	4.9	3.5	1.4	1.6	57.4		

It appears from these experiments that the essential factor responsible for the "shock" is the dilution of the serum proteins which is effected by the plasma depletion. The body cells cannot tolerate this diluted medium which for them is an abnormal environment. Protoplasmic injury is readily proved and if this injury is too extensive we note a familiar sequence of events which ends with fatal "shock." One may point out the narrow line which delimits a mild injury due to this plasma dilution from a severe or lethal injury and at times the reaction almost approaches the "all or none law." The change in urinary nitrogen following a moderate reaction and plasma depletion may be almost zero but following a severe or almost fatal shock due to plasma depletion we may observe a rise in urinary nitrogen on the day following which amounts to 100 to 200 per cent increase over nor-

mal. This indicates a serious injury of protein substance in the body. In a fatal plasmapheresis we may note a rapid increase in the blood non-protein nitrogen which may show over 100 per cent rise within 3 to 4 hours.

PLASMAPHARESIS COMPLICATED BY KNOWN TISSUE INJURY

In the large table 21 are collected a number of experiments to show that the presence of *injured liver cells* will predispose an animal to severe or lethal shock following a control or standard plasmapheresis. The control experiments show little or no shock following the plasma depletion of a given volume. But the same exchange performed after chloroform or phosphorus usually results in fatal shock. These experiments are in contrast to those in table 28, which presents the results of plasma depletion associated with cell injury of the kidney, pancreas and intestine. Injured cells of these organs do not modify the reaction following a standard plasmapheresis.

The three following experiments (tables 22, 23 and 24) illustrate in detail the reaction which follows plasmapheresis when preceded by chloroform anesthesia to insure a certain amount of liver necrosis and injury. The first of this group (table 22) gives a control plasmapheresis to prove that the plasma depletion alone was not responsible. The amount of liver injury was not extreme and could be tolerated by any normal animal with no clinical reaction. Note other experiments with controls in table 21.

The emergency reaction which makes possible a rapid replacement of the washed out serum proteins shows in all these experiments. The presence of the injured liver and the development of fatal shock does not modify the usual reaction by which a considerable amount of serum proteins is thrown into the circulation. This may suggest that this reaction is not purely a functional reflex but perhaps a physical phenomenon in which we see a simple exchange of protein between body cells and the circulating blood plasma—a simple washing out of a given substance related to the serum proteins which is normally present in certain body cells.

TABLE 21

Liver injury predisposes to fatal shock after plasma depletion

DOG NUMBER	POISON	BLOOD VOLUME EX- CHANGE		SHOCK	BLOOD SERUM PROTEINS IN PER CENT				REMARKS
		Per cent	Time in minutes		Before exchange	End of exchange	15 minutes after	24 hours after	
17-212	0	141	12	None	5.5	2.6	2.5	3.9	
17-212	Chloroform (1 hour)	144	13	Fatal	5.5	2.1	3.4		
18-6	0	118	17	Slight	7.1	4.2		4.8	
18-6	Chloroform (1½ hour)	175	12	Fatal	6.6	1.7	2.5		
18-9	0	170	12	Moderate	5.6	2.0	2.9	4.2	
18-9	Chloroform (1½ hour)	198	10	Severe	4.8	2.0	2.4	4.0	Drug given 48 hours previ- ously
17-215	0	159	10	Slight	6.2	1.3	2.2	4.1	
17-215	Phosphorus (17.5 mgm.)	140	7½	Fatal	4.9	1.2	1.2		Drug given 40 hours previ- ously
17-233	0	67	5½	Slight					
17-233	Phosphorus (14 mgm.)	118	15	Fatal	4.9	0.7	2.1		
18-34	0	75	6	Moderate	5.3	2.9	3.4	4.3	
18-34	Phosphorus (5.2 mgm.)	77	6½	Fatal	5.6	3.1	3.5		
18-7	0	96	12	None	5.5	2.7	3.6		
18-7	Phosphorus (11 mgm.)	118	7	Moderate	6.5	1.4	3.2	3.7	Drug given 5 hours later
18-66	0	91	9	Moderate	6.2	3.2	3.8	4.3	
18-66	Hydrazine (140 mgm.)	82	9	Fatal	5.7	4.2			
18-68	0	90	9	Moderate	5.5	2.8	3.5	4.8	
18-68	Hydrazine (100 mgm.)	88	9	Moderate	4.9	2.6	3.3	4.6	
18-68	Hydrazine (100 mgm.)	95	7	None	5.2	2.3	3.1		

Poison given in every experiment 18 to 26 hours before plasmapheresis unless otherwise noted.

Plasmapheresis before and after chloroform

Experiment 71. (See table 22). 198 per cent exchange.

Dog 18-9. Female bull-terrier pup. Weight 12 pounds.

August 2. Plasmapheresis, 170 per cent exchange in 12 minutes. Little if any intoxication.

August 4. Chloroform anesthesia for 1½ hour, undergoing recovery without clinical signs of injury.

August 6. Animal appears to be in excellent condition. Under ether anesthesia 1081 cc. of blood were withdrawn from the left femoral artery. At the same time and at the same rate 1081 cc. of Locke's corpuscle suspension were injected into the left femoral vein. The exchange was effected in a space of 10 minutes. There was a steady fall in blood pressure and in the volume of the pulse. An extreme grade of depression was present within a half-hour and the heart beat was barely palpable 3 hours after the exchange. The rectal temperature had fallen at this time to a level of 30°C. From this point on slow but gradual improvement was noted. Eventually complete recovery occurred.

TABLE 22

198 per cent blood volume exchange; plasmapheresis following chloroform; dog 18-9; experiment 71

TIME	BLOOD SERUM READINGS IN PER CENT				REMARKS
	Total protein	Albumin	Globulin	Non-protein	
Before exchange.....	4.8	3.2	1.6	1.7	Profound shock
Immediately after.....	2.0	1.2	0.8	1.3	
15 minutes after.....	2.4	1.5	0.9	1.6	
2nd day.....	4.0	2.4	1.6	2.3	Good recovery
3rd day.....	4.9	3.1	1.8	2.0	

Plasmapheresis following chloroform

Experiment 72. (See table 23). 175 per cent exchange.

Dog 18-6. Young-adult female Dachshund. Weight 13.4 pounds. Blood volume on July 1 (by dye method) was 761 cc.

On July 24 a 118 per cent exchange was performed in 8 minutes with little or no shock.

August 7. Chloroform anesthesia for 1½ hour.

August 8. Under ether anesthesia 1065 cc. of blood were withdrawn from the left femoral artery. Simultaneously an equal quantity of Locke's corpuscle suspension was injected into the left femoral vein. The duration of the exchange was 12 minutes. The rectal temperature showed little immediate alteration as a result of the exchange. However a gradual fall in temperature soon appeared, the level of 36.4°C. being reached at the time of death. 1½ hour later. The arterial pulse became slow and weak almost at once following the exchange. The respiration was gasping in character within 15 minutes following the exchange

and a profound degree of depression existed. The condition gradually became worse and death occurred $1\frac{1}{2}$ hour following the exchange.

Autopsy: The blood drawn from the heart shows no tendency to clot within the space of 24 hours. Even such blood when placed in contact with fresh tissues shows no tendency to clot. The thoracic organs are negative. The spleen is somewhat enlarged and the Malpighian bodies are approximately twice their normal size. The pancreas is slightly congested. The liver is congested. A considerable amount of necrosis due to chloroform injury is seen in the centers of the lobules. Histological examination shows a fairly extensive central hyaline necrosis involving about one-half of each liver lobule. There is some fatty de-

TABLE 23

175 per cent blood volume exchange; plasmapheresis following chloroform; dog 18-6; experiment 72

TIME	BLOOD SERUM READINGS IN PER CENT				REMARKS
	Total protein	Albumin	Globulin	Non-protein	
Before exchange	6.6	3.6	3.0	1.7	Death
Immediately after	1.7	0.6	1.1	1.6	
15 minutes after	2.5	1.5	1.0	1.6	
$1\frac{1}{2}$ hours after					

TABLE 24

144 per cent blood volume exchange; plasmapheresis following chloroform; dog 17-212; experiment 78

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMO-GLOBIN PER CENT (SAHLI)	REMARKS
	Total protein	Albumin	Globulin	Non-protein			
Before exchange	5.5	3.1	2.4	1.6	0.33	97	Death
Immediately after	2.1	1.4	0.7	1.5	0.13	92	
15 minutes after	3.4	2.4	1.0	1.3	0.18	92	
2 hours	5.5	3.6	1.9	1.7	0.12	93	

generation of the liver cells in the mid-zone of each lobule. This lesion could be tolerated by a dog with few if any clinical symptoms. The kidneys show considerable engorgement of the medulla. The mucosa of the entire gastro-intestinal tract is pink and a considerable amount of mucus is contained in the lumen.

Plasmapheresis following chloroform

Experiment 78. (See table 24). 144 per cent exchange.

Dog 17-212. Young adult female spaniel. Weight 14.5 pounds. Blood volume on July 19 (by dye method) was 632 cc.

An exchange of 94 per cent was performed on July 23, and another of 141 per cent on August 1, with practically no signs of shock in either case.

August 21. Chloroform anesthesia for 1 hour.

August 22. Animal appears to be in excellent condition. Under ether anesthesia 950 cc. of blood were withdrawn from the left carotid artery. Simultaneously 950 cc. of Locke's corpuscle suspension were injected into the left external jugular vein. The duration of the exchange was 13 minutes. The rectal temperature gradually fell about 4°C. from the normal level in the 2 hours following the exchange. Fluid blood-stained feces were noted at the end of the first hour following the exchange. The dog went into profound shock and died 2 hours following the exchange.

Autopsy: The thoracic organs are negative. The spleen and kidneys show considerable congestion. The liver is large and congested. In gross there is evidence of chloroform injury and histological sections show an early stage of chloroform necrosis which involves liver cells in the centers of lobules. This injury is slight in degree and by itself would give no clinical reaction in the dog. The mucosa of the stomach and small intestines is thickened and dark red in color. A considerable excess of mucus and fluid material is present in the intestinal lumen.

The following experiment (table 25, exper. 95) is complete in that a control plasmapheresis causes only a little intoxication. The dose of phosphorus is less than one-half a lethal dose and would be tolerated by a normal dog without clinical symptoms. The combined phosphorus injury and a second plasmapheresis causes a typical lethal shock.

It may be noted that the *hematocrit* figures which are complete for this experiment show no evidences of any definite change in red cell plasma ratio. The same observation holds in the chloroform experiments. When we review all these shock experiments and compare them with duplicate experiments in which no shock appears we cannot assign any of these reactions to a process of concentration of the blood. In certain experiments there is a rise in cell hematocrit taken 15 minutes and 1 to 4 hours after the exchange. But the same rise is noted at the very end of the exchange and the correct explanation we believe is to be found in the red cell mixture introduced. This red cell mixture contains more red cells per cubic centimeter than the blood of the dog under observation. There is a constant fall of hematocrit on the 2nd day but we believe this is to be explained by the disintegration of the red cells which have been injured in the routine process of washing in Locke's solution.

The second phosphorus experiment (no. 73) is given in table 21. The control plasmapheresis caused no reaction but the same exchange

preceded by a small dose of phosphorus was fatal in 2 hours. In another experiment the plasmapheresis was *followed* by a large dose of phosphorus. The intent was to follow the curve of protein regeneration as influenced by this drug which causes such characteristic liver injury.

Plasmapheresis following phosphorus

Experiment 95. (See table 25). 77 per cent exchange.

Dog 18-34. Female bull pup. Weight 17.1 pounds. Blood volume (by dye method) was 805 cc.

September 5. The usual plasmapheresis with 80 per cent exchange was completed in 4 minutes without the production of shock.

September 26. Plasmapheresis with 75 per cent exchange was carried out in 6 minutes. There was a certain amount of clinical depression, but no serious shock.

TABLE 25

77 per cent blood volume exchange; plasmapheresis following phosphorus; dog 18-34; experiment 95

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMA- TOCRIT RED CELL PER CENT	REMARKS
	Total protein	Al- bumin	Globu- lin	Non- protein			
Before exchange.....	5.6	4.2	1.4	2.0	0.16	55	
Immediately after....	3.1	2.3	0.8	1.3	0.26	54	
15 minutes after.....	3.5	2.6	0.9	1.6	0.33	58	
3 hours.....	3.6	2.6	1.0	1.7	0.30	54	
6 hours.....	4.0	2.9	1.1	1.5	0.29	54	Profound shock

October 16. Phosphorus, 5.2 mgm. in olive oil, given subcutaneously.

October 17. Animal appears to be in excellent condition. Under ether anesthesia 600 cc. of blood were withdrawn from the right carotid artery. Simultaneously 600 cc. of Locke's corpuscle suspension were injected into the right external jugular vein. The duration of the exchange was 6.5 minutes. The arterial tension was fair at the end of the exchange, but was very poor at the end of another half-hour. Although showing a considerable amount of prostration, the animal was conscious for several hours. The animal was in profound shock at the end of 6 hours, and was found dead 12 hours after the exchange. The body was still somewhat warm, but rigor mortis was fairly well developed.

Autopsy: The tissues at the root of the lungs and about the smaller bronchi within the lung are somewhat edematous. The spleen is practically normal. The liver is quite pale and slightly translucent. Its lobulation is indistinct. Kidneys show slight congestion along the cortico-medullary line. The stomach is negative. The mucosa of the small intestine is thickened and moderately congested. A considerable amount of mucus is found in the lumen.

Histological sections: The liver shows very little evidence of cell injury. There are a few pale nuclei, but the fatty change so common in the cell protoplasm after large doses of phosphorus is absent. A slight increase in the leucocytes in the liver capillaries is noted. Spleen, pancreas, lung and intestines are negative.

Plasmapheresis following phosphorus

Experiment 73. 140 per cent exchange.

Dog 17-215. Young adult female fox-terrier. Weight 17.4 pounds. Blood volume on July 19 (by dye method) was 858 cc.

July 31. An exchange, 159 per cent, was performed in 10 minutes, causing no definite signs of shock.

August 7. Phosphorus, 17.5 mgm. in olive oil, was given subcutaneously.

August 9. Under ether anesthesia 1105 cc. of blood were withdrawn from the left femoral artery. Simultaneously 1105 cc. of Locke's corpuscle suspension were injected into the left femoral vein. The duration of the exchange was 8 minutes. Profound depression was in evidence almost immediately. There was a very marked weakening in the pulse. Bloody feces appeared within an hour following the exchange. Death followed the exchange by 2 hours. There was a gradual fall in rectal temperature of 4°C. during the course of the experiment. The total blood serum proteins fell from 4.9 per cent to 1.2 per cent as a result of this exchange. Other figures are not available because of loss of material.

Autopsy: Blood drawn from the heart immediately after death does not clot even on the addition of tissue juices. The thoracic organs are negative. The spleen is dark red and enlarged to about twice the normal size. The Malpighian bodies are large, distinct and opalescent. The liver is somewhat enlarged. The centers of the hepatic lobules are dull red while the peripheral portions are yellowish. The stomach shows distention of the superficial veins and moderate engorgement of its mucosa. The mucosa of the duodenum and upper portion of the jejunum is markedly engorged. The mucosa of the lower portion of the small intestine is but slightly reddened, while the large intestine is negative. The pyramids of the kidneys are slightly engorged. A few scars are seen in the cortex.

Histological sections: Liver shows early changes in cell protoplasm, especially small fat droplets. This dose of phosphorus should give a severe but not lethal liver injury. The injury at this stage is very inconspicuous. There is a notable interstitial edema of the pancreas. Other organs are negative.

Plasmapheresis followed by phosphorus

Experiment 65. (See table 26). 118 per cent exchange.

Dog 18-7. Young adult female mongrel terrier. Weight 10.9 pounds. Blood volume (by dye method) was 530 cc.

July 19. An exchange, 96 per cent, performed in 12 minutes caused no shock.

July 26. Under ether anesthesia 583 cc. of blood were withdrawn from the femoral artery. Simultaneously 583 cc. of Locke's corpuscle suspension were

injected into the femoral vein. The duration of the exchange was 7 minutes. With the exception of a fall of about 1°C. in rectal temperature there was little obvious disturbance as a result of the exchange. About 5 hours after the exchange 11 mgm. of phosphorus dissolved in olive oil were injected subcutaneously. On the following day the animal appeared rather quiet, but not otherwise disturbed. The food was not eaten for several days and on August 1 the dog weighed 9.25 pounds. Complete recovery occurred several days later.

TABLE 26

118 per cent blood volume exchange; plasmapheresis followed by phosphorus; dog 18-7; experiment 65

TIME	BLOOD SERUM READINGS IN PER CENT			
	Total protein	Albumin	Globulin	Non-protein
Before exchange.....	6.5	5.7	0.8	2.0
Immediately after.....	1.4	0.3	1.1	1.5
15 minutes after.....	3.2	1.8	1.4	1.7
2nd day.....	3.7	2.6	1.1	2.5
3rd day.....	3.8	2.4	1.4	2.3
5th day.....	5.1	2.0	3.1	1.5
10th day.....	4.3	2.0	2.3	1.8

TABLE 27

88 per cent blood volume exchange; plasmapheresis following hydrazine sulfate; dog 18-68; experiment 108

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATO-CRIT RED CELL PER CENT
	Total protein	Albumin	Globulin	Non-protein		
Before exchange.....	4.9	3.2	1.7	1.7	0.31	27
Immediately after.....	2.6	1.7	0.9	1.6	0.26	42
15 minutes after.....	3.3	2.3	1.0	1.4	0.31	56
3½ hours.....	3.7	2.6	1.1	1.9	0.40	41
2nd day.....	4.6	3.2	1.4	1.7		
3rd day.....	4.7	3.3	1.4	1.6		33
6th day.....	5.2	2.8	2.4	2.3		

Plasmapheresis following hydrazine sulfate

Experiment 108. (See table 27). 88 per cent exchange.

Dog 18-68. Female mongrel bull pup. Weight 14.3 pounds.

November 21. An exchange of 90 per cent performed in 9 minutes caused a moderate grade of shock.

November 27. Hydrazine sulfate, 100 mgm., injected subcutaneously.

November 28. Under ether anesthesia 575 cc. of blood were withdrawn from the left femoral artery. Simultaneously 575 cc. of Locke's corpuscle suspension were injected into the left femoral vein. The duration of the exchange was 9

minutes. There were at no time any definite signs of depression. The arterial tension remained moderately good throughout. There was a fall in rectal temperature of about 1°C. during the exchange. There was, however, a prompt return—in fact to a point slightly above the original temperature for a period of several hours, after which the temperature returned to the normal level.

The preceding experiment (table 27) gives some evidence that hydrazine sulfate as a liver poison differs somewhat when compared with chloroform or phosphorus. This dog (18-68) showed no less reaction to the control plasmapheresis than to the same exchange preceded by hydrazine sulfate. In another experiment, however, (table 21, exper. 109) we see the familiar reaction with fatal shock due to a combined plasmapheresis and hydrazine poisoning. The control of the plasmapheresis showed a definite but not severe intoxication.

The preceding table (table 28) lists the reactions which follow a plasmapheresis combined with cell injuries of various other organs and tissues. The control plasma depletion on the same dog is given in each experiment. When the remarkable reaction and fatal shock were noted in the phosphorus and chloroform experiments we suspected at once that any cell injury might render the experimental animal more sensitive to the shock of plasmapheresis. The experiments in table 28, however, show that such is not the case.

The *kidney* epithelium was injured by administration subcutaneously of uranium nitrate in suitable dosage. Two experiments show identical reactions in the control plasma depletion as in the plasmapheresis following the administration of uranium nitrate. One experiment (dog 18-35) shows a fatal reaction but there are many unusual features which we cannot explain—see table 30 below for details.

Pancreas injury is represented by only a single experiment but this is very clean-cut. The pancreas was injured by the injection of bile into its main duct. The control exchange gives the same negative reaction as the plasma depletion preceded by the acute pancreatitis.

The *Roentgen-ray* is able to cause a specific and extensive injury to the lymphatic tissue but especially to the *epithelium* of the *small intestine* as has been shown by the work of Hall and Whipple (6). This injury and consequent intoxication develops to its maximum on the 4th day following an exposure over the abdomen. A plasmapheresis done 24 hours after X-ray exposure gives the same reaction as in the control period. This shows that even the extensive injury which in a fatal case of X-ray intoxication involves the greater part of the epithelium of the small intestine does not modify the shock of plasmapheresis. This is in striking contrast to the liver injury.

TABLE 28

Kidney, pancreas and intestinal epithelium injury does not predispose to shock after plasma depletion

DOG NUMBER	POISON	BLOOD VOLUME EXCHANGE		SHOCK	BLOOD SERUM PROTEINS IN PER CENT			
		Per cent	Time in minutes		Before ex- change	End of ex- change	15 minutes after	24 hours after
18-35	0	74	14	None	5.5	3.7	3.8	
18-35	Uranium (5 mgm.)	72	14	Fatal	6.3	3.9	4.6	5.8
18-48	0	104	2	Slight	6.4	2.1	3.2	4.5
18-48	Uranium (6 mgm.)	77	4	None	6.1	3.1	4.1	4.6
18-66	0	91	9	Moderate	6.2	3.2	3.8	4.3
18-66	Uranium (8 mgm.)	96	11½	Moderate	5.1	2.9	3.2	4.8
18-65	0	100	13	Slight	5.6	3.0	2.9	4.6
18-65	Pancreatitis	94	15	None	5.3	3.3	3.9	4.8
18-68	0	90	9	Moderate	5.5	2.8	3.5	4.8
18-68	X-ray* (175 M.A.M.)	90	7	Moderate	5.9	3.2	3.9	4.7
18-65	0	100	13	Slight	5.6	3.0	2.9	4.6
18-65	X-ray (200 M.A.M.)	105	9	None	5.7	2.9	3.3	4.7

Injury given in every experiment 20 to 24 hours before plasmapheresis with exception noted: * X-ray given 45 hours before plasmapheresis.

TABLE 29

77 per cent blood volume exchange; plasmapheresis following uranium nitrate; dog 18-48; experiment 99

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATO- CRIT RED CELL PER CENT
	Total protein	Albumin	Globulin	Non- protein		
Before exchange	6.1	3.4	2.7	1.7		
Immediately after	3.1	1.9	1.2	1.4		
15 minutes after	4.1	2.6	1.5	1.5	0.25	61
2 hours	4.0	2.6	1.4	1.5	0.25	36
6 hours	4.7	2.9	1.8	1.8	0.32	29
2nd day	4.6	3.0	1.6	2.0	0.45	41
6th day	5.2	3.4	1.8	2.2	0.42	41
8th day	5.1	3.4	1.7	2.2	0.29	46
9th day	5.1	3.4	1.7	2.1		
11th day	5.2	3.5	1.7	2.4	0.33	43

Plasmapheresis following uranium nitrate

Experiment 99. (See table 29). 77 per cent exchange.

Dog 18-48. Female bull pup. Weight 14.3 pounds.

October 4. Exchange of 99 per cent performed in 12 minutes with the production of but slight grade of shock.

October 18. An exchange of 104 per cent in 2 minutes was performed with very little shock.

October 31. Uranium nitrate, 6 mgm., given subcutaneously.

November 1. Animal seems to be in excellent condition. Under ether anesthesia 500 cc. of blood were withdrawn from the right carotid artery. Simultaneously 500 cc. of Locke's corpuscle suspension were injected into the right external jugular vein. The duration of the exchange was 14 minutes. At no time during the experiment was there any definite sign of shock. The arterial tension was good at the end of the exchange but shortly thereafter fell slightly for a period of several hours. The rectal temperature fell nearly 2°C. during the exchange but returned to normal in the course of several hours.

Plasmapheresis before and after uranium nitrate

Experiment 97. (See table 30). 72 per cent exchange.

Dog 18-35. Female bull pup. Weight 16.7 pounds.

September 12. An exchange of 80 per cent was performed in 7 minutes, causing slight intoxication.

October 3. An exchange of 73 per cent was effected in 14 minutes, with no signs of intoxication.

October 22. An aqueous solution of 10.4 mgm. *uranium nitrate* was injected subcutaneously.

October 23. Animal appears to be in good general condition. Under ether anesthesia 545 cc. of blood were withdrawn from the left carotid artery. Simultaneously and with no great variation in rate 545 cc. of Locke's corpuscle suspension were injected into the left jugular vein. The duration of the exchange was 13 minutes. The rectal temperature fell from 39.5 to 37°C. within a space of 4 hours. Drowsiness soon appeared, the pulse diminished in volume after about 3 hours and was decidedly poor several hours later. Bloody feces were first noted about 11 hours following the exchange. The next morning the condition was worse and a considerable amount of bloody feces had been passed. The animal was suffering from convulsive attacks. The temperature was 37°C. Death occurred about 20 hours following the exchange.

Autopsy: The tissues are definitely jaundiced. The thoracic organs are essentially negative. The spleen is large, the edges rounded, and on section presents definite congestion. The liver shows only indistinct lobulation. The kidneys are definitely engorged. The stomach is negative except for a slight amount of engorgement of the mucosa. The intestinal mucosa is decidedly engorged with blood, the condition being more marked in the lower portion of the gut.

Histological sections: Kidneys show much epithelial degeneration and necrosis involving particularly the convoluted tubules. There are numerous hyaline, hemoglobin and blood casts in the collecting tubules. Other organs present nothing of interest.

The preceding experiments (tables 29 and 30) are in conflict. The first one (exper. 99) shows a negative reaction when a standard plasma depletion is combined with kidney injury due to uranium nitrate. The other experiment (exper. 97) shows a fatal reaction but it is atypical. The shock did not develop quite as usual and the dog seemed about to recover. When the shock of plasmapheresis is tolerated for 12 hours the dog usually recovers and appears normal and active within 24 hours. This dog on the day after the experiment developed convulsions and died. There was jaundice and at autopsy signs of blood destruction. The histological sections give evidence of considerable epithelial injury in the secreting tubules of the kidney. That this kidney injury played a part in the late death is highly probable but

TABLE 30

72 per cent blood volume exchange; plasmapheresis following uranium nitrate; dog 18-35; experiment 97

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATOCRIT RED CELL PER CENT	REMARKS
	Total protein	Albumin	Globulin	Non-protein			
Before exchange.....	6.3	4.7	1.6	1.7	0.32	22	
Immediately after.....	3.9	3.0	0.9	1.5	0.14	25	
15 minutes after.....	4.6	3.5	1.1	1.4	0.16	42	
2½ hours.....	5.1	3.7	1.4	1.4	0.19	38	
5 hours.....	5.2	3.9	1.3	1.4		37	
2nd day.....	5.8	4.0	1.8	1.8	0.32	30	Death

it is clear that the shock of plasmapheresis was atypical. In view of the other experiments we do not attach too much importance to a single atypical experiment which appears to be at variance with the general type reaction.

Acute pancreatitis followed by plasmapheresis

Experiment 102. (See table 31). 94 per cent exchange.

Dog 18-65. Female bull pup. Weight 17 pounds.

November 7. An exchange of 100 per cent effected in 13 minutes caused a very moderate grade of shock.

November 13. Under ether anesthesia laparotomy was performed and 10 cc. of sterile bile injected by means of a hypodermic needle into the pancreatic duct. The wound was closed. It is known that this will cause an intense diffuse hemorrhagic pancreatitis.

November 14. The animal is lively and apparently in quite good condition. Under ether anesthesia 730 cc. of blood were withdrawn from the left femoral artery. Simultaneously 730 cc. of Locke's corpuscle suspension were injected into the left femoral vein. Seven minutes were consumed in effecting the exchange. At no time was there any definite evidence of intoxication. Refer to experiment 106, table 33, for autopsy.

The preceding experiment (table 31) is complete and supports the two following experiments with X-ray injury. This pancreas was severely injured by an injection of bile into the pancreatic duct. We have frequently produced an acute hemorrhagic pancreatitis in this way and the injury may be sufficient to produce lethal intoxication by itself. That extensive injury was done this pancreas is afforded by

TABLE 31

94 per cent blood volume exchange; acute pancreatitis followed by plasmapheresis; dog 18-65; experiment 102

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATO-CRIT RED CELL PER CENT
	Total protein	Albumin	Globulin	Non-protein		
Before exchange	5.3	3.6	1.7	2.2	0.49	36
Immediately after	3.3	2.5	0.8	1.6	0.29	54
15 minutes after	3.9	2.9	1.0	1.4	0.22	58
3 hours	4.6	3.1	1.5	1.4	0.40	53
8 hours	4.3	2.9	1.4	2.2	0.34	41
2nd day	4.8	2.9	1.9	2.2	0.29	40
3rd day	4.6	2.6	2.0	2.2	0.25	35
4th day	4.0	2.8	1.2	2.8	0.35	32
6th day	5.0	2.8	2.2	2.9	0.31	27
7th day	5.0	3.7	1.3	2.7	0.40	29

examination of the autopsy record of this dog (exper. 106, table 33 below) which shows a scarred, indurated pancreas speckled with old fat necroses. Yet these injured pancreas cells did not modify the reaction following a controlled plasma depletion.

Plasmapheresis following sublethal X-ray exposure

Experiment 111. (See table 32). 90 per cent exchange.

Dog 18-68. Female bull pup. Weight 14 pounds.

November 21. An exchange of 90 per cent effected in 9 minutes caused a very moderate grade of shock.

December 10. X-ray exposure over abdomen in 4 quadrants, 2 mm. aluminum filter, 175 milliamperes minutes, with 9 inch spark gap. Distance from target to skin is 10 inches.

December 12. Animal seems to be in excellent condition. Under ether anesthesia 575 cc. of blood were withdrawn from the left carotid artery. Simultaneously 575 cc. of Locke's corpuscle suspension were injected into the left external jugular vein. The duration of the exchange was 7 minutes. There was but a very slight amount of depression. The arterial tension was good at the end of the exchange, but became of poorer quality in the course of the next 2 hours. The animal walked about occasionally. The rectal temperature fell about 1°C. during the course of the experiment.

Lethal dose of X-ray followed by plasmapheresis

Experiment 106. (See table 33). 105 per cent exchange.

Dog 18-65. Female bull pup. Weight 16.8 pounds.

November 7. An exchange of 100 per cent effected in 13 minutes caused a very moderate grade of shock.

November 14. An exchange of 94 per cent following acute experimental pancreatitis produced no definite signs of intoxication (see exper. 102, table 31).

November 21. X-ray exposure over abdomen in 4 quadrants, 2 mm. aluminum filter, 200 milliamperes minutes, with 9 inch spark gap. Distance from target to skin is 10 inches.

November 22. The animal appears to be in good condition. Under ether anesthesia 800 cc. of blood were withdrawn from the right carotid artery. Simultaneously 800 cc. of Locke's corpuscle suspension were injected into the right external jugular vein. The duration of the exchange was 9 minutes. The animal showed a very slight amount of depression as a result of the exchange. The pulse remained fair throughout. There was but slight depression of the rectal temperature (1°C.).

November 23. The animal seems a bit weak. Has vomited material containing some intestinal worms.

November 24. The dog appears somewhat better.

November 25. Quite weak. Mucous, blood-tinged feces. Some vomiting. Refuses food.

November 26. Death occurred in the afternoon. Autopsy performed at once.

Autopsy: Thoracic organs negative. Blood drawn from the heart clots normally. Blood urea nitrogen is 32.5 mgm. per 100 cc. Spleen is small, light red, with an increase in fibrous tissue. Liver is pale and anemic. Pancreas: lower arm is hard, shrunken and scarred; the result of the pancreatitis described in experiment 102, table 31. Many old fat necroses are present. The upper arm is scarred but appears more nearly normal. Kidneys and adrenals are negative. Gastro-intestinal tract shows only a few scattered patches of congestion.

Histological sections: The pancreas shows extensive fibrosis—the result of the preceding acute injury. The small intestine shows much epithelial injury in its deep crypts. There is some evidence of epithelial regeneration as well as degeneration. This epithelial injury we believe to be the immediate cause of death. Other organs present nothing of interest for this experiment.

The two preceding experiments (tables 32 and 33) show the influence of X-ray injury of the body cells upon a standard plasmapheresis.

The first experiment (table 32) shows the result of a sublethal exposure to the X-rays. The reaction to the plasma depletion in a control exchange is not modified.

The second experiment (table 33) shows a reaction recently described in some detail by Hall and Whipple (6). This reaction is due

TABLE 32

90 per cent blood volume exchange; plasmapheresis following sublethal x-ray exposure; dog 18-68; experiment 111

TIME	BLOOD SERUM READINGS IN PER CENT				REMARKS
	Total protein	Albumin	Globulin	Non-protein	
Before exchange.....	5.9	4.2	1.7	2.2	No shock
Immediately after.....	3.2	2.6	0.6	1.7	
15 minutes after.....	3.9	3.0	0.9	1.8	
3 hours.....	4.4	3.1	1.3	1.9	
5½ hours.....	4.5	3.3	1.2	1.9	
2nd day.....	4.7	3.3	1.4	2.7	Dog normal
4th day.....	4.5	3.0	1.5	2.6	

TABLE 33

105 per cent blood volume exchange; lethal dose of x-ray followed by plasmapheresis; dog 18-65; experiment 106

TIME	BLOOD SERUM READINGS IN PER CENT				FIBRIN IN PER CENT	HEMATOCRIT RED CELL PER CENT	REMARKS
	Total protein	Albumin	Globulin	Non-protein			
Before exchange.....	5.7	3.7	2.0	2.1	0.45	33	Slight shock
Immediately after.....	2.9	2.3	0.6	1.7	0.22	44	
15 minutes after.....	3.3	2.6	0.7	1.7	0.27	34	
4 hours.....	4.1	2.8	1.3	1.8	0.28	37	
7 hours.....	4.2	3.4	0.8	1.5			
2nd day.....	4.7	3.5	1.2	2.1	0.47	26	X-ray intoxication
3rd day.....	3.9	2.1	1.8	3.0		25	
4th day.....	4.0	1.7	2.3	3.8	0.72	30	
5th day.....	5.9	3.0	2.9	3.1	0.95		Death

to a lethal dose of the X-ray—in this instance 200 milliampere minutes, 90 kilo volts, given over the abdomen. Death on the 4th day with the usual blood-tinged feces and prostration is the usual reaction in these animals given a lethal exposure of the X-ray. Details of this reaction and the post-mortem findings may be found in the publica-

tion just noted. The control plasmapheresis which was done 24 hours after the X-ray exposure did not give any symptoms of intoxication and this reaction due to the plasma depletion was not modified by the presence of a great amount of injured epithelium of the small intestine. We have many experiments to show that on the second day after X-ray exposure *epithelial injury and necrosis* can be made out histologically in the small intestine. These cells will undergo rapid autolysis under a variety of conditions and it is quite remarkable that the plasmapheresis should not be modified by this great mass of injured epithelial cells.

DISCUSSION

In some earlier experiments, Kerr, Hurwitz and Whipple (3), it was noted that the presence of liver injury or liver cell necrosis made a given animal much more vulnerable to the injury and consequent shock which followed a given plasma depletion. Using single rapid depletion by the method described in the experiments cited above similar results were observed. A number of such experiments are given in table 21 above and it will be noted that the control experiment in every case shows little or no shock following plasmapheresis, but an identical procedure if associated with slight liver injury was almost always fatal. There is apparently little or no difference in this respect between the liver injury due to chloroform and that due to phosphorus. The liver injury due to hydrazine sulfate was not studied in a sufficient number of experiments.

The interesting fact stands out that a trifling injury due to phosphorus or chloroform can be tolerated by a dog with no clinical reaction. But if at this time (24 to 48 hours after administration of the chloroform or phosphorus) we perform a plasmapheresis of small volume which was previously tolerated by the same dog with little or no intoxication, we immediately precipitate severe or fatal shock. The combination of slight liver injury and a moderate exchange (plasmapheresis) will result fatally in almost all cases. How may we explain this observation? There are many possibilities but we favor the following explanation. The chloroform or phosphorus causes an injury to many liver cells and these cells are more susceptible to other injurious agents than are the normal liver cells. A sudden change in the protein content of the blood which bathes these injured cells will react more unfavorably upon them than upon the healthy and more resistant normal liver cells. These damaged (phosphorus) and then shocked (plas-

mapharesis) liver cells form substances which are taken up by the blood and carried to all the living cells of the body. If these poisonous substances are sufficient in amount we observe the development of lethal shock. We may not assume simple intensive injury and paralysis of the *liver cells alone* because it is known that the body can tolerate complete ablation of the liver cells for a period of 5 to 7 hours (7). When we produce an intensive form of shock (plasmapharesis) we may observe death within 1.5 hour. This cannot be explained by any *local reaction* limited strictly to the liver cells.

We observe in other experiments (table 28) that cell injury of other organs (kidney, pancreas and intestine) does not modify the familiar reaction following a moderate exchange. The control and poisoning experiments give similar reactions. This indicates a peculiar relation of the liver cells to the *shock reaction* associated with plasma depletion.

SUMMARY

Bleeding a dog from a large artery and a simultaneous replacement of a red blood cell Locke's solution mixture may be called "plasma depletion" or "plasmapharesis." This procedure will rapidly wash out large amounts of plasma proteins and cause a precipitous fall in the blood plasma protein concentration.

The reaction following such procedures may be minimal or it may be lethal. In general the larger the exchange the greater the probability of lethal shock. Repeated plasma depletions carried out at intervals of days or weeks on the same animal will give uniform reactions if the volume exchange and other experimental factors are constant.

"Plasmapharesis" may be performed with washed red cells suspended in normal dog serum or fresh dialyzed dog serum. When we replace the Locke's solution in the red cell mixture by dog serum we remove completely the toxic effect of the plasma depletion. This gives control for the experimental procedure but, more important, gives strong indication that the *blood serum proteins are stabilizing or protective factors*. They are essential environmental factors of the circulating blood in its relation to the body cells. This may be the most important function of these plasma colloids.

The presence of injured cells of the kidney, pancreas or intestine does not seriously modify the expected reaction following a uniform plasmapharesis.

The presence of *injured liver cells* (chloroform, phosphorus) *does profoundly modify the expected reaction* following a unit plasmapheresis. A fatal shock reaction is almost constant following even a moderate plasma depletion preceded by liver injury.

This would indicate that the liver cells are particularly concerned in the peculiar shock reaction which may follow plasmapheresis and lowering of the blood plasma protein values. It may be that this type of "shock" is not unlike the common "surgical shock."

The evidence in our experiments gives strong support to the theory that in "shock" there is a *primary cell injury* which *precedes* the familiar *clinical reaction*.

BIBLIOGRAPHY

- (1) MORAWITZ: Beitr. z. chem. Physiol. u. Path., 1906, vii, 153.
- (2) ABEL, ROWNTREE AND TURNER: Journ. Pharm. Exper. Therap., 1914, v, 625.
- (3) KERR, HURWITZ AND WHIPPLE: This Journal, 1918, xlvii, 356, 370, 379.
- (4) KERR, HURWITZ AND WHIPPLE: This Journal, 1918, xlvii, 356.
- (5) ERLANGER AND GASSER: This Journal, 1919, xlix, 151.
- (6) HALL AND WHIPPLE: Amer. Journ. Med. Sci., 1919, clvii, 453.
- (7) WHIPPLE AND HOOPER: Jour. Exper. Med., 1913, xvii, 612.

III. FACTORS CONCERNED IN THE PERFUSION OF LIVING ORGANS AND TISSUES

ARTIFICIAL SOLUTIONS SUBSTITUTED FOR BLOOD SERUM AND THE RESULTING INJURY TO PARENCHYMA CELLS

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These experiments were undertaken for the purpose of investigating the formation of the serum proteins in the body. The source of the serum proteins is still a mystery in spite of indirect evidence brought out by Kerr, Hurwitz and Whipple (1) to show that the *liver* is concerned in the regeneration of new serum proteins as well as in the maintenance of the normal serum protein concentration in the circulating blood. It seems too that other organs or tissues as well as the liver must be able under emergency conditions to produce certain amounts of new serum protein. It would appear that the normal wear and tear of serum proteins must be slight as these substances can be formed only with so much difficulty when the normal level has been greatly lowered.

Theoretically, perfusion of organs should offer an ideal method of solving these problems. Using a mixture of red cells and whole serum or diluted serum or modified Locke's solution, the investigator should be able to perfuse satisfactorily the various organs or combinations of organs and tissues. A simple determination of the protein values before and after such a procedure would then give the desired information and enable one to say whether a certain organ did or did not contribute any serum proteins. Information as to whether serum proteins are used by or destroyed in these organs might also be made available.

We have been able to convince ourselves that the present methods are not satisfactory to permit of the solution of this serum protein problem just outlined above. One of the conclusions which has been forced upon us is that much of the older experimentation in the field of organ perfusion is of little or no value as regards deductions made from

such experiments which postulated a living organ or organ cells. When parenchymatous organs are perfused with Locke's solution or some modification of this solution with or without red cells, we wish to suggest the probability that the research worker is dealing not with normal cells but with cells which have been injured or destroyed by contact with the perfusate. The investigator is then perfusing a dying or dead autolysing tissue or organ. Deductions drawn from such experiments must be cautious and proper allowance in every case made for this profound injury of the parenchyma cells.

The "stabilizing value" of the blood serum proteins and the injury done the various body cells by contact with diluted plasma have been emphasized in the preceding communication. We may also note the observation of Guthrie (2) to the effect that organ transplantation is a failure if the transplanted organ is washed out with normal saline before the blood flow through the organ is reestablished. The transplanted organ does not resume its function and we may assume that its cells were definitely injured by the short period of contact with the salt solution.

The gradual slowing of the perfusion flow through any given organ is a familiar observation and we believe it may be due in part to actual injury of cells, including the endothelium. The simultaneous development of edema is a part of the same general reaction.

From many of our earlier experiments we gained the impression that perfusion or plasmapheresis with the use of Locke's solution inflicted a destructive injury upon certain body cells and that this injury of protein was responsible for the fatal intoxication and death. Our conception was somewhat as follows: the initial injury might damage irreparably liver cells (or other body cells) and from these injured organ cells were derived poisonous substances (protein split products) sufficient to cause death. It has been pointed out that complete paralysis of all liver cells could not explain this phenomenon as ablation of the liver can be tolerated for 5 to 7 hours, while the shock following a large plasmapheresis may cause death within 2 hours.

Postulating the presence in the body of some poisonous substance referable to the perfusion or plasmapheresis, we have attempted in many of our perfusion experiments to demonstrate the presence of a poison in the perfusate at the end of any given experiment. In only one experiment (exper. 5) have we evidence for the presence of any poison under these conditions of perfusion. But we have some evidence (expers. 17 and 18) to show that a known poison of protein origin

added to a given non-toxic perfusate may be in part removed within 20 to 30 minutes' continuance of perfusion. This is evidence that a poison of colloid nature may be removed from the circulation in such experiments—so the absence of a poison in our perfusates does not negative the possibility of poison being formed by the injured cells and contributed to the blood stream or perfusate. We have further been able to show that an enormous dose of proteose-like, toxic material may be wholly removed from the blood stream within a period of 5 minutes after intravenous injection in a normal dog.

We have discussed the results of the experiments given below in the light of the plasmapheresis experiments given in the two preceding papers. It may be noted that these perfusion experiments were done *before* the plasma depletion experiments. The evidence which may be taken from our perfusion experiments is not as definite as that obtained in the later plasmapheresis experiments but these data are all in harmony. A most important fact is that *physiological perfusion* of organs is very difficult and *slight modification of the blood plasma* may have profound effect upon body cells.

DEVELOPMENT OF PERFUSION METHODS

The notion of artificial perfusion was long ago expressed by Le Gallois (3). He maintained that by artificial perfusion life might be kept up in any portion of the animal even though separated from the rest of the body. It remained, however, for other workers actually to undertake such experiments. In 1828 Kay (4) showed that artificial perfusion with blood was capable of restoring irritability to dying muscle. Artificial perfusion of kidneys was first attempted in 1849 by Löbell (5). The work of Brown-Séquard (6) done several years later showed the necessity of oxygenation of the blood used as a perfusate. The oxygenated blood was forced through the arteries by means of a syringe. In this manner he perfused various regions including the isolated head. He found that he was able in this manner to maintain certain evidences of reflex nervous activity provided the perfusion was commenced promptly after decapitation. Ludwig and Schmidt (7) in 1868 described an apparatus by means of which blood could be forced under constant pressure from a reservoir. Improvements in aeration of the perfusion medium were made by Schröder (8). Fry and Gruber (9) devised an artificial lung by means of which the aeration of the perfusate could be accomplished without interrupting the flow of blood

to the region being perfused. Although fluctuation in the pressure supplied to the perfusion medium occurred in the work of the earlier investigators using the syringe injection method, the distinct beneficial effects of such variations in pressure were first recognized by Ludwig and Schmidt (7). Fry and Gruber (9) attached the piston of a syringe supplying the arterial pressure to a motor-driven wheel thus creating by mechanical means a pulsatile pressure. Hamel (10) emphasized the need for pulsatile pressure. He devised an apparatus in which the movements of a pendulum periodically interrupted the flow of the perfusate to the tissues, thus converting a constant pressure into an intermittent one. Jacobj (11) devised an elaborate perfusion apparatus in which pulsatile pressure was created by periodic and forcible compression of a rubber balloon placed in the arterial side of the circuit. He used the principle of aerating the blood by forcing a mixture of air and venous blood through a stretch of tubing at the end of which the blood and air were separated by gravitation. In a later paper Jacobj (12) described a method by means of which the blood was aerated by perfusion through a lung in which respiration was artificially maintained. In this manner he avoided the direct mixing of the blood with the air. In 1903 Brodie (13) published an account of an apparatus which has subsequently been used by several investigators. With it he was able to perfuse an organ with the use of no other blood than that obtained from the animal itself—a considerable advantage over many of the types previously employed. To create pulsatile pressure he suggests that a fairly distensible piece of rubber tubing placed in the arterial side be rhythmically compressed by a wooden arm.

Other forms of perfusion apparatus have been described by Hoffmann (14), Richards and Drinker (15), Friedmann (16), Mandel (17) and Kingsbury (18).

Pulse pressure as a necessary factor in the mechanics has been recently reemphasized by Hooker (19), whose apparatus was employed in our experiments. His apparatus can be adjusted in such a manner as to furnish a pulse curve identical in form to that produced in a normal intact animal. Aeration is effected.

In addition to the purely mechanical methods of perfusion another slightly different procedure has been employed by some. As far back as 1881 Martin (20) attempted to study the activity of the heart by diverting all of the blood issuing from the aorta back into the right auricle. A heart-lung preparation was thus effected, the circulation being successfully excluded from the rest of the body. This procedure

or modifications thereof have been used by many workers since that time. In 1914 Bainbridge and Evans (21) substituted this living preparation for the artificial perfusion machine. The organ to be perfused received its blood directly from the aorta of the preparation. The venous blood issuing from the vein was returned to the right side of the heart. The perfused tissue thus received blood aerated by the lungs and under pulsatile pressure supplied by the heart itself. It should be remarked that in this form of perfusion the study of the perfused organ is complicated by the metabolism of the heart and lungs themselves.

EXPERIMENTAL OBSERVATIONS

All perfusion experiments were performed by use of the apparatus designed and described in detail by Hooker (19), (22). Through his courtesy we were able to obtain this machine which was made after the model of his original apparatus. We take this opportunity to acknowledge our appreciation for valuable assistance on the part of Dr. D. R. Hooker. The general experimental procedures are covered by the brief description in individual experiments. All experiments were done on dogs under complete ether anesthesia. In all perfusion experiments the dog was placed upon a warm pad to keep up the body temperature.

Perfusion of hind legs with Locke's solution

Experiment 5. Male bull pup. Weight 4.8 kilos.

Under ether anesthesia cannulae were inserted and the hind legs were perfused for $\frac{1}{2}$ hour with Locke's solution. The temperature of the perfusate varied between 30° and 40°C. The perfusion pressure was between 100 and 110 mm. mercury. The pulse pressure was between 20 and 30 mm. mercury. Pulse rate 130 a minute. The 240 cc. of perfusate recovered at the end of the perfusion contained 77,000 red blood cells per cubic millimeter. Hemolysis was moderate in amount.

To test the toxicity of this perfusate the cells were removed by centrifugalization and 100 cc. of the supernatant fluid were injected intravenously into a normal dog. A rise of 1.4° in temperature with vomiting and diarrhea was noted. The pulse was not markedly altered. The perfusate was therefore moderately toxic.

Ten cubic centimeters of the centrifugalized perfusate were also injected intraperitoneally into a 100 gram rat. Slight toxicity was evident.

Perfusion of hind legs with red corpuscles suspended in Locke's solution

Experiment 7. Male collie mongrel. Weight 16 pounds.

The hind legs were perfused with a suspension of blood corpuscles in Locke's

solution. The temperature of the perfusate varied between 32° and 38°C. The mean pressure was 50 mm. mercury. The pulse rate was 130 and the rate of flow 180 cc. a minute.

Autopsy shows irregular petechial hemorrhages in the muscles and fascia of the hind legs.

Slight hemolysis was noted in the 250 cc. of perfusate recovered at the end of perfusion. One hundred and sixty-one cubic centimeters of the centrifugalized end-product were injected intravenously into a normal 13.75 pound dog. A temperature rise of 0.4° was observed. No vomiting or diarrhea occurred. Twenty cubic centimeters of this perfusate were also injected intraperitoneally into a rat weighing 100 grams. There were no evidences of toxicity from the use of this perfusate.

These two experiments (expers. 5 and 7) give little information concerning the actual perfusion conditions but supply data concerning the production of a poison by the Locke's perfusion. There is slight positive evidence for a toxic reaction in experiment 5 but a negative reaction in experiment 7. In general we have no distinctly positive evidence that this destructive perfusion of body tissues will give demonstrable evidence of a toxic element in the perfusate. As stated above this may be explained by the capacity of the body cells to remove such poisons from circulating fluids.

Perfusion below diaphragm with red corpuscles suspended in modified Locke's solution

Experiment 9. Normal female black and white pup. Weight 6.75 pounds.

The oxalated blood from a normal dog was centrifugalized and the corpuscles washed in gelatin-Locke's solution minus calcium by mixture and recentrifugalization. The red cells were then suspended in gelatin-Locke's minus calcium in the ratio of packed corpuscles 1 to solution 5. This mixture was used as the perfusion medium.

Under ether anesthesia the animal was bled. Cannulae, were inserted and the perfusate forced through the aorta and recovered from the right auricle, thus perfusing the area below the diaphragm. The temperature of the perfusate varied between 35° and 38°C. The mean pressure was maintained at 50 to 80 mm. mercury; the pulse pressure between 10 and 15 mm. mercury. The pulse rate was 130 a minute. The flow was excellent. Perfusion began 20 minutes after bleeding and was continued for 1 hour.

The autopsy was delayed for a few hours after the completion of the perfusion. The muscles of the hind legs were pale and showed very little edema. No hemorrhages were present in the muscles and connective tissue. The liver was normal except for some air bubbles in its vessels. The capsule of the kidneys stripped readily. Hyperemia was seen at the cortico-medullary boundary. Suprenals were negative. The lymph nodes of the mesentery were normal.

The pancreas showed a considerable amount of edema. The spleen was dark red except for a transverse light band possibly caused by block from emboli. The intestines were filled with red mucoid material. The mucosa was velvety, swollen and deep purplish-red in color. Histological sections of liver and intestine give no evidence of tissue abnormality.

On centrifugalization a sample of perfusate taken 10 minutes after beginning of perfusion was light pink. A sample taken at the end of 40 minutes was somewhat deeper in color, while a sample taken at the end of perfusion was dark red.

One hundred and fifty cubic centimeters of a centrifugalized sample taken after 10 minutes of perfusion were injected into a small normal dog. The animal vomited once. There was a rise of 1°C . in temperature. The pulse remained good. No marked symptoms of intoxication were present.

Eight cubic centimeters of the 10-minute sample were injected intraperitoneally and 2 cc. were injected subcutaneously into a 75-gram rat. No toxic action was noted. This test was repeated by injecting 5 cc. intraperitoneally into a 38-gram rat. No toxic action was noted. The same amount of the 40-minute perfusate sample was injected intraperitoneally into a 62-gram rat. No toxic action.

Perfusion below diaphragm with red blood cells in modified Locke's solution

Experiment 11. Female shepherd pup. Weight 2280 grams.

In preparation of the perfusate blood corpuscles were obtained from the blood of a normal dog bled several hours previously into a 1 per cent sodium oxalate solution. The red cells were centrifugalized and washed in Rous' gelatin-Locke's solution minus calcium. The packed cells were then suspended in a similarly prepared calcium-free gelatin-Locke's solution in the ratio of one part of corpuscles to five of the saline mixture.

Under ether anesthesia the animal was bled and the cannulae were arranged to perfuse all of the tissues below the diaphragm. Ten minutes were consumed in arranging the cannulae. The temperature of the perfusate was between 35° and 38°C . The pulse pressure was about 10 mm. mercury. Due to clots in the gauze two stops were necessitated over a period of 5 minutes each. One occurred soon after the beginning of perfusion and one some minutes later. The duration of the perfusion was 1 hour. During this period the animal increased 760 grams in weight.

Autopsy showed about 75 cc. of pale bloody fluid in the abdominal cavity. Marked edema was present about the pancreas and throughout the mesentery. Hemorrhagic spots were observed over the surface of the kidney and stomach, about the ovary and throughout the muscles and fascia of the hind legs. The liver on section was translucent. On section the kidney showed indefinite dark hemorrhagic spots up to 0.5 cm. in diameter. The whole organ was dark and congested. The stomach contents were normal. Mucus and bloody fluid were present in the small intestine. Congestion and bloody intestinal contents were more prominent in the lower part of the small intestine.

The perfusate showed a moderate grade of hemolysis before perfusion but less after 15 minutes of perfusion. Moderate hemolysis existed at the end of the perfusion.

Bacteriological cultures showed 500 bacteria per cubic centimeter in samples taken at end of perfusion.

Two hundred and eight cubic centimeters of the perfusion fluid obtained at the end of the perfusion were injected intravenously into a normal small dog. With the exception of a temperature rise of 1.5° and some shivering there were no signs of intoxication.

The two experiments (expers. 9 and 11) show the results of a perfusion of all the organs and tissues below the diaphragm by a red cell Locke's solution mixture. It is to be noted especially that there is marked edema of retroperitoneal tissues and the pancreas. This edema is invariably present in considerable amount except when whole defibrinated blood is used as perfusate. We accept the edema as one indication of tissue or cell injury. The same is true of hemorrhagic areas and ecchymoses, but some of them may be due to emboli. The marked congestion of the intestinal mucosa with the escape of blood-tinged fluid and mucus is also a valuable index of injury. This is a familiar reaction noted in dogs dead from anaphylaxis or large doses of proteose or from surgical shock. The perfusate in both these experiments contained no poisonous substance for normal dogs and white rats.

Hemolysis is always present in slight or moderate degree in all our experiments. We are inclined to explain a part of this hemolysis by the cell injury in organs or tissues and this cell injury reacts unfavorably upon the red cells with resulting hemolysis. We realize that the dog's red corpuscles are most fragile and that the red cells are subjected to much mechanical injury in these experiments. Other observers may choose to explain all this hemolysis upon a purely traumatic basis.

Perfusion below diaphragm with diluted defibrinated blood

Experiment 13. Male bull pup. Weight 2150 grams.

Two parts of defibrinated blood obtained from a normal dog were diluted with one part of gelatin-Locke's solution. The animal was anesthetized with ether, the thorax opened and the cannulae inserted in such a way that the perfusion medium was forced into the aorta just above the diaphragm and the blood received from the inferior vena cava just below the heart. In this way the entire region below the diaphragm was perfused. The temperature of the perfusion medium was maintained at about 32° to 38°C . The systolic pressure varied from 95 to 120 mm. mercury with a pulse pressure of 20 mm. mercury. The return flow was accidentally occluded for a few seconds at the beginning of the experiment. The perfusion lasted 1 hour.

Examination of the region perfused showed hemorrhagic streaks in the diaphragm and gall bladder. Numerous small areas of hemorrhage accompanied by a considerable amount of edema existed about both kidneys. The liver showed considerable congestion and edema. The lobules of the pancreas were

distinctly separated by edema. The spleen was quite dark. The stomach showed considerable engorgement with sub-mucous hemorrhages. Externally the small intestines were spotted by numerous small subserous hemorrhages. The intestinal mucosa showed diffuse congestion while the lumen contained a little dark mucus. The colon was more nearly normal in appearance; however, a few small sub-serous hemorrhages were seen. The hind legs showed no hemorrhage or edema. There was a weight increase of 400 grams.

The perfusate showed slight hemolysis before perfusion and a moderate grade of hemolysis at the end of perfusion. The perfusate obtained at the end of perfusion contained 39 mgm. of non-protein nitrogen and 16 mgm. of urea-nitrogen per 100 cc.

TABLE 34

Perfusion below the diaphragm with defibrinated blood. Experiment 16

TIME	COLOR OF CENTRIFUGAL- IZED PERFUSATE	CARBON DIOXIDE CAPACITY PER 100 CC.	HYDROGEN ION CONCENTRA- TION	REMARKS
Before.....	Pale pink	38.5	7.5	More blood added
After 20 minutes.....	Rose	15.7	7.3	
After 40 minutes.....	Rose	26.1		
At the end.....	Deep rose	12.8	7.4	

Perfusion below the diaphragm with defibrinated blood

Experiment 16. Normal male bull pup. Weight 4.2 pounds.

Under ether anesthesia cannulae were inserted for perfusion below the diaphragm. This region was perfused for 30 minutes with pure defibrinated blood at a rate of 100 cc. per minute. The cannulae were then shifted to the lower abdominal vessels and the hind legs perfused at a rate of 25 cc. a minute for 60 minutes, with the same perfusate. The perfusate was aerated with pure oxygen and was maintained at a temperature varying from 36° to 39°C. The pulse rate was 141 a minute.

Intestinal peristalsis was quite conspicuous at the beginning of perfusion but was less noticeable after the perfusion had been in progress for about 5 minutes. During this period the abdominal wall was very sensitive to touch and contracted violently when touched.

At autopsy a large amount of clear straw-colored fluid was noted in the abdominal cavity. The animal had gained 200 grams in weight as a result of the perfusion. The liver was slightly translucent although apparently normal. The spleen was somewhat congested. The pancreas showed no edema. The kidneys were slightly congested in the pyramidal areas. The stomach was normal. The duodenum was likewise normal but the ileum showed a mucosa congested and dark red with grey mucoid material in the lumen. The hind legs showed no edema and were quite dry in appearance. A few petechial hemorrhages appeared in the fascia.

Histological sections: Pancreas and kidney are normal. The spleen and liver show capillary congestion but normal parenchyma cells. The stomach and small intestine are normal except for slight congestion of the ileum.

In these two experiments (experiments 13 and 16) we used diluted or whole defibrinated blood. The general autopsy picture following the use of whole blood is almost normal and the lack of the edema we believe is to be explained on this ground. Even in the last experiment we note the development of ascites which of course indicates circulatory abnormality. There is further a distinct acidosis to be explained by inadequate aeration of the blood.

Perfusion with whole defibrinated blood—toxic proteose added

Experiment 17. Adult male poodle dog. Weight 10.25 pounds.

Under ether anesthesia the animal was bled. The arterial cannula was placed just above the bifurcation of the iliaes. The venous cannula was inserted in the inferior vena cava just above the renal veins. Thirty minutes were consumed in bleeding, arranging the cannula and starting the perfusion flow. The hind legs were perfused for 32 minutes with defibrinated blood. To the 500 cc. of perfusate then remaining in the apparatus 100 cc. of a *proteose solution* (lethal dose is 2 cc. per pound body weight, adult dog) were added and the perfusion continued for 12 minutes. The perfusate was warmed to a temperature of 35° to 38°C. The rate of flow was 80 cc. per minute until near the end of the experiment, when it decreased to 50 cc. per minute. The aeration of the perfusate was excellent. The proteose solution was prepared as described elsewhere (23) from the material of the obstructed intestine.

Autopsy of the perfused dog showed slight icterus of the tissues of the hind legs but no hemorrhages or edema. A few small hemorrhages were found in the right testicle. The pelvic organs were negative.

At the end of perfusion there were obtained 300 cc. of perfusate for analysis. Of this were injected into a 14-pound normal pup, 227 cc., which represented 38 cc. of the original proteose. This would be a fatal dose for an adult dog weighing 19 pounds, provided no proteose had been lost. Pups are more susceptible to proteose intoxication than adults. This dog therefore received a theoretical dose of one and one-half times its lethal dose, assuming that no proteose was lost from the perfusate during the perfusion.

The injection of 227 cc. of perfusate caused death in 3 hours with the clinical picture of acute proteose intoxication. Diarrhea and vomiting appeared within $\frac{1}{2}$ hour after injection and continued until death. There was an initial rise in temperature followed by a drop to 36.8°C. half an hour before death. Autopsy findings showed exquisite splanchnic engorgement especially marked in the mucosa of the small intestines, which was a velvety purplish-red coated with mucus—described and pictured elsewhere (24).

The results of examination of the perfusate are given in the table below (table 35).

Perfusion with whole defibrinated blood—toxic proteose added

Experiment 18. Under ether anesthesia a small female mongrel dog was bled. Cannulae were inserted preparatory to perfusing all of the body below the diaphragm. Defibrinated blood warmed to 35° was perfused through this region under a mean pressure of 110 mm. of mercury. The pulse pressure varied between 5 and 15 mm. of mercury. The pulse rate was 120 per minute. Aeration of the perfusate was not quite as satisfactory as usual. Actual perfusion commenced 30 minutes after bleeding the animal. After the flow has been maintained for 20 minutes 100 cc. of proteose solution were added to the 250 cc. of defibrinated blood then remaining in the apparatus. Perfusion was continued for 15 minutes. The rate of flow was 150 cc. a minute at first, but gradually decreased to 85 cc. a minute toward the end of the experiment. Marked intestinal peristalsis was present during the first few minutes of perfusion; blood-tinged feces later.

Autopsy showed a moderate quantity of pale blood-tinged fluid in the peritoneal cavity. The animal had gained 180 grams in weight during the experiment. The liver showed edema and small hemorrhages throughout. Numerous small hemorrhages as well as several larger ones were seen in the wall of the gall bladder. The pancreas was negative except for one small hemorrhage. The mesenteric lymphatics contained blood-stained fluid. The adrenals contained several small hemorrhagic areas. The kidneys were negative. The spleen was small, dark and translucent. The stomach showed one fairly large but no small hemorrhages. The mucosa of the duodenum was engorged. The intestinal lumen contained an excess of thin bloody fluid with little mucus. It is possible that some of the hemorrhages noted above are to be explained by short periods of high blood pressure during the periods of perfusion.

As was stated above, 100 cc. of the proteose solution (lethal dose is 2 cc. per pound body weight, adult dog) were added to the 250 cc. of perfusate then in circulation and the perfusion continued for 15 minutes. Of the final perfusate 77 cc. were injected intravenously into a normal 11-pound pup (no. 17-181). It is evident that these 77 cc. contained 22 cc. of the original proteose solution provided none of this toxic material had been removed during perfusion by the tissues of the animal perfused. The reaction of the animal might be expected therefore to be lethal, if no proteose was removed during the perfusion through the tissues of the first dog. The reaction to this intravenous injection was typical for a *sublethal* toxic dose of proteose. There was vomiting and diarrhea for 2 hours and much prostration. Recovery was evident in 3 hours and the dog was normal in a few more hours. It appears that some of the proteose had been removed as the amount given was more than a lethal dose for a pup of 11 pounds body weight.

The final perfusate was further examined and shown to contain definite amounts of hemoglobin (hemolysis). Bacteriological examination (plates) showed 40,000 colonies per cubic centimeter of the perfusate. The non-protein nitrogen at beginning of perfusion was 34.7 mgm. per 100 cc. of perfusate and at the end was 35.8 mgm.

In these two experiments (expers. 17 and 18) the perfusion was done with whole defibrinated blood to insure a minimum injury of the perfused tissues. After the initial perfusion a standardized toxic solution was added to the perfusate and again circulated as before. The perfusate at the end of the experiment was tested on a normal dog and in this way it was demonstrated that some of the poison had been removed. It is easy to show that large doses of this toxic proteose are rapidly removed from the circulation of a dog. Following intravenous injection of large amounts of toxic proteose it is possible to demonstrate its presence for 2 to 3 minutes in the blood stream but not after 5 minutes. The presence of bacteria as noted in this and other experiments will not seriously disturb the reaction. If anything, their presence will increase the toxicity of the perfusate mixture. These bacteria probably gain

TABLE 35

Perfusion with whole defibrinated blood—toxic proteose added. Experiment 17

TIME	HEMOLYSIS	PER CENT BLOOD CELLS	UREA NITROGEN	NON- PROTEIN NITROGEN	HYDROGEN ION	CARBON DIOXIDE CAPACITY
			<i>mgm. per 100 cc.</i>	<i>mgm. per 100 cc.</i>		
Before perfusion.....	Moderate	47	19	26	7.5	16
After one-half hour....	Moderate	50	16	31	7.4	16
On adding proteose....	Moderate	47	18	30	7.4	19
At end of perfusion....	Slight	45	20	89	7.4	13

entrance in part through the intestinal tract and in part are added by the manipulation of the perfusate. Efforts were made to preserve the circulating machinery in as near a sterile condition as possible but there are many possibilities for introducing contamination.

The "proteose solution" used in these experiments is prepared from material obtained from obstructed intestines or closed intestinal loops in dogs. The material is precipitated by five volumes of alcohol, the precipitate dissolved in water and the protein removed by boiling in dilute acetic acid solution. A second precipitation with alcohol is often employed. The final solution is an opalescent fluid which contains proteose-like materials. This fluid material is then standardized by intravenous injection in normal dogs, as has been described elsewhere (23).

Large infusion of Locke's solution into portal vein

Experiment 21. Dog 17-125. Normal adult mongrel, black and tan. Weight 43 pounds.

Under ether anesthesia and with sterile precautions the abdomen was opened and the hepatic-pancreatico-duodenal and pancreatico-duodenal arteries were ligated. Sterilized calcium-free Locke's solution, 1750 cc., was warmed to approximately 36°C. and injected at a rate of 55 cc. a minute into the portal system through a small venous branch in the mesentery. The abdomen was closed. The animal showed no severe reaction for some time but died 3 days later.

Autopsy performed several hours later shows considerable post-mortem change. Dark red softened areas containing bubbles of gas are seen scattered throughout the liver. The serosa of the intestines is quite red. The mucosa shows moderate engorgement and is covered by a buttery exudate. The coil of intestine to which the perfused vein was distributed differs in no way from the other parts of the small intestine. Kidneys are negative. The total non-protein nitrogen of the blood shows but slight alteration from the original value as a result of the experimental procedure.

Histological sections: The picture is somewhat confused by post-mortem changes but it is clear that there are scattered areas of liver cell necrosis which are ante-mortem and presumably related to the experimental procedure. These areas include many liver lobules and present a uniform necrosis with scattered leucocytes between the liver cell strands. Bile ducts and blood vessels are normal and no evidence of vascular thrombosis is observed in any sections. Other sections show normal liver parenchyma. Other tissues present nothing of importance.

Large infusion of Locke's solution into splenic artery

Experiment 23. Dog 17-200. Young male collie. Weight 29.5 pounds. Normal except for a slight attack of distemper.

Under ether anesthesia and with sterile precautions the abdomen was opened. A cannula was inserted into the splenic artery in such a manner that the upper arm of the pancreas, a part of the duodenum and stomach, as well as the liver, were perfused by 2000 cc. of sterile calcium-free Locke's solution injected at a rate of 50 cc. a minute. The saline was injected at room temperature. Splenectomy followed the perfusion. The pancreas showed moderate edema at the end of the experiment, but the dog was not severely shocked. Later a state of intoxication slowly developed and at the end of 36 hours death was imminent. The animal was killed by ether.

Autopsy performed at once shows a little blood-stained peritoneal fluid. The liver shows a moderate grade of cloudy swelling. The subserous tissues of the gall bladder are thick and edematous. A number of sub-serous ecchymoses are scattered over the small intestine. Several hemorrhagic areas are present in the mucosa of the small intestine. Hemorrhages and fat necrosis are rather pronounced in the upper arm of the pancreas.

Histological selections: Organs are normal with the exception of the pancreas and liver. The pancreas shows extensive hemorrhagic necrosis and much nec-

rosis of fat and gland parenchyma. There are no thrombus masses noted in any of the vessels. The head of the pancreas is essentially normal. The liver shows scattered clumps of polymorphonuclear leucocytes and evidence of injury to small clusters of liver cells in various portions of the liver lobules.

Large infusion of Locke's solution into the portal vein

Experiment 24. Dog 17-203. Adult male mongrel. Weight 29.5 pounds. Slight distemper.

Under ether anesthesia the abdomen was opened and the hepatic artery clamped. One of the larger splenic veins was isolated, a cannula inserted and 1500 cc. of sterile calcium-free Locke's solution warmed approximately to body temperature were injected into the portal system over a period of 18 minutes. The clamps were then removed from the hepatic artery. Splenectomy was performed and the abdomen was closed. The temperature rose to 40°C. shortly after the operation and the animal vomited once; otherwise no clinical disturbance was noted. Complete recovery ensued. The animal was killed 7 days later. The autopsy was negative. No alteration could be made out in the liver.

To obviate the mechanical difficulties inherent in organ perfusion and to get information concerning the direct effect of Locke's solution upon tissue and organ cells we performed a number of experiments of which experiments 21, 23 and 24 are examples. The first experiment (exper. 21) shows a fatal reaction following a large infusion in the portal vein after ligation of the branches of the hepatic artery to limit the blood flow through the liver. We have explained this reaction as due in part to injury of the liver cells by contact with the mixtures of Locke's solution and blood. It is known that ligation of the hepatic artery will cause no disturbance in the dog. It seems hard to account for these areas of liver necrosis except as due to the action of the portal blood diluted by the large infusion of Locke's solution into the portal vein. It is to be noted (exper. 24) that a similar experiment was tolerated without obvious liver injury but the occlusion of the hepatic artery in this experiment was only temporary.

The pancreas necrosis was surely caused by the perfusion of the splenic artery (exper. 23). It may be objected that perfusion in this way against the arterial stream will cut off the tissues from oxygen by washing away all available red cells. We are inclined to believe that arterial collaterals which are numerous in this region will insure the presence of the necessary number of oxygen-carrying red cells. This objection cannot apply to experiment 21.

DISCUSSION

In attempting an analysis of our own experiments we wish to draw freely on the published work of other investigators. We wish to keep constantly in the reader's mind that *physiological perfusion* of any organ is a matter of extreme difficulty and often great confusion is introduced by such methods which are intended to simplify the study of organ function. By *physiological perfusion* we mean a perfusion adequate to maintain the organ in its normal physiological activity.

In the first place let us inquire what criteria of tissue abnormality we have. What sort of evidence is going to lead us to pass judgment concerning the physiological condition of tissues? There are certain conditions under which we may ascertain what is going on in the tissues by a direct observation of the functional activity of the part perfused. For example, in perfusing the kidney the quantity and quality of the urine secreted furnishes some evidence concerning the condition of that organ. The reduction within the organ of oxyhemoglobin to hemoglobin was noted by the earliest observers and is indicative of metabolic activity of some nature on the part of the perfused tissues. The nature of the heart beat is indicative of the condition of the perfused heart, although Magnus (25) has shown that if such an inert substance as hydrogen gas be perfused through the coronary arteries heart beats will be stimulated. Sollmann (26) showed that the same result followed perfusion with cottonseed or paraffin oil. In such cases the perfusion fluid cannot be thought of as being a nutrient fluid; on the other hand it is not altogether impossible that, as was suggested by Sollmann, the heart beats may be stimulated by purely mechanical factors. For these reasons we must not hastily conclude that, because the gross mechanical movements simulate those occurring in the intact animal, the preparation is in fact an example of normal physiological activity.

A criterion as to the condition of the perfused medulla is furnished by observing whether the medulla continues to maintain its normal control over the heart and muscles of respiration.

In addition to direct observation of the functional activity of an organ perfused, we have still other kinds of evidence which help us to judge concerning the condition of the tissue. Thus the rate of flow through the vessels is in some cases a valuable indicator for it is a general rule that tissue injury brings about in some way or other a decrease in the rate of flow through the part. We have, in addition, the still more crude signs of tissue injury: edema, congestion and hemorrhage.

Of the factors in the procedure of perfusion whose variations might bring about injury to the part, the following may be mentioned as being perhaps the most important: aeration of the perfusion medium, composition of the perfusion medium, interruptions in continuity of flow, temperature of the perfusion medium when it enters the perfused organ, mean pressure and pulse pressure.

It should be realized that a perfusion experiment is no better than its weakest point. If any of the above factors react in such a way as to cause injury to an organ, perfection of the other factors will not remedy the defect. It is also conceivable that when several organs are being perfused simultaneously, injury to one organ or tissue may react injuriously on others.

Concerning the effect on the tissues of composition of the perfusate, the literature contains many references to condition of the tissues as shown by functional activity. The injurious effects of foreign blood have been known since the time of Prévost and Dumas (27) when this fact first began to be recognized through the failure of foreign blood to act normally after transfusion. Though repeatedly shown to be harmful in its effects on tissues of another species, foreign blood has been used in perfusions even as late as Brodie (13) who says that ox, sheep or horse blood cannot be used in the perfusion of organs taken from dogs. He finds that as soon as foreign blood is supplied to the perfused heart the beat becomes irregular. The heart next goes into fibrillary twitchings and cannot be recovered from this state even with the animal's own blood.

Although defibrinated blood had been used in the transfusion experiments of Prévost and Dumas (27) without the observation of harmful results, Magendie found it incapable of carrying on the normal function of the circulating medium after transfusion. In a series of experiments in 1822 (28) and again in 1838 (29) he presented experimental data to show that the lack of fibrin, reduced through repeated bleeding, defibrination and reinjection of the defibrinated blood gives rise to a serous and bloody transudate into the lungs and intestine with the death of the animal.

The weight of Magendie's name behind such a statement did much to discredit defibrination in the eyes of other workers, but eventually Biscoff (30), Goll (31), Polli (32), Panum (33), Ponfick (34), and many others began to turn the weight of experimental evidence against a belief in the extreme toxicity of defibrinated blood when used in transfusions between animals of the same species

More recently, by means of perfusion experiments, Stevens and Lee (35) and Brodie (13) present evidence of slight vasoconstriction due to the use of defibrinated blood as a perfusion medium. Their work again points to an injurious action of defibrinated blood when substituted for the normal circulating medium. However, Stevens and Lee believe that the slight vascular contraction which they note can be readily counteracted with pharmacological agents.

In 1903 Pfaff and Vejux-Tyrode (36) found defibrinated blood definitely injurious to the kidney of the dog. Quantities of from $\frac{1}{4}$ to $\frac{1}{10}$ of the total blood were withdrawn from the carotid artery, whipped, filtered and reinjected into the jugular vein. The repetition of this procedure resulted in the appearance of albumin, hemoglobin and red blood cells in the urine and finally cessation of secretion. However, a rapid return to normal was effected in these animals by bleeding followed by direct transfusion of whole blood from a normal dog. It would seem that the kidney may be unusually sensitive to this procedure.

It is certain that in those of our animals which underwent quite complete defibrination (37)—(see expts. 323 and 324)—there were no clinically evidenced signs of injury or toxic manifestations. This may also be said of those experiments of Whipple and Goodpasture (38) in which quite complete defibrination was also effected.

The importance of a physiological balance of the normal inorganic salts of the blood is generally recognized. Solutions containing abnormal quantities of these salts have been shown to be toxic to the perfused heart. Hooker (22), (39) showed that in perfusion of the respiratory center a balance of potassium and calcium is essential for a normal function of the preparation. As was early shown by several investigators and more recently reemphasized by Hooker (19) and by others, and as we have found in our experiments, saline solution has the property of setting up such a condition in the tissues that the rate of flow gradually decreases.

The effects of variations in composition of perfusion media may also be manifest from the morphological side. Brodie (13) shows that edema results from the use of foreign blood. Hamel (10) shows that edema results in organs perfused with saline under pulsatile pressure. Similarly the kidney when perfused with Locke's solution exhibits more edema than when perfused with defibrinated blood (19). These results are entirely in accord with our experience. Perfusion with pure Locke's solution almost invariably produces an extreme grade of edema. Dog's defibrinated blood diluted with Locke's solution produced less,

and pure defibrinated blood produces very little demonstrable edema. It is difficult to explain just why dilution with Locke's solution should produce edema. In several of our experiments we carried out hydrogen-ion and carbon dioxide determinations on the perfusate and it is interesting to note that in cases of marked edema there was a rise in the hydrogen ion concentration and a fall in the carbon dioxide capacity. Whether the edema is the result of the acidosis or not, there still remains the question as to what is the cause of the decrease in the buffer substances. Perhaps it may be attributable in part at least to insufficient oxidation in the tissues. Poor oxidation may result from insufficient oxygen-carrying capacity of the perfusate or from inadequate aeration of the perfusate in the artificial lung, or may result from a decreased rate of flow through the animal and a stagnation of the blood in the tissues with consequent asphyxia.

A factor which has been shown to be of considerable importance in causing tissue injury is that of loss of time in establishing the artificial perfusion after interrupting the normal relations. Most of the earlier workers paid but scant attention to this phase of the problem. In many experiments several hours elapsed before any attempt was made to reestablish the flow. Grube (40) mentions that in perfusion of the liver with defibrinated blood to which glucose had been added, the glycogen content of the liver rises, but only in case the circulation is very promptly reestablished. The deleterious effect on the kidney of temporary anemia is well known. Momentary compression of the renal vessels may cause a cessation of secretion for many minutes. The effect of compression of the cerebral vessels has been known since very early times. The duration of such anemia necessary to produce irreparable damage was long ago studied by Astley Cooper (41). Signs of activity can be restored to the brain of the isolated head provided only that the perfusion is promptly commenced (6), (42), (43) and (44). Skeletal muscle is capable of surviving much longer periods of anemia than is the case of brain or kidney. Munk (45) holds that in perfusion of the kidney if the flow is not promptly commenced the vessels become narrowed, the perfusion flow rendered difficult and a delay in the formation and flow of urinary fluid occurs. Recently Bainbridge and Evans (21) have succeeded in perfusing the kidney without any interruption whatsoever in the continuity of flow.

A great deal has been said in the literature concerning the value of pulsatile pressure. As has already been noted, Ludwig and Schmidt (7) observed that with constant pressure the rate of outflow from the

perfused tissue decreases, but that recovery occurs if the flow is halted for a while. Similar observations were subsequently made by many workers. The recognition of the importance of this factor is evidenced by the numerous forms of apparatus devised to accomplish this end. Hooker (19) holds that in the perfused kidney the amount of urinary filtrate formed varies directly with the magnitude of the pulse pressure. The amount of proteins in the urinary filtrate varies inversely with the magnitude of the pulse pressure. The rate of blood flow through the organ varies directly with the magnitude of the pulse pressure. Recently Gesell (46) has been able in the intact animal to abolish almost completely the pulse pressure in the renal arteries without interfering with the normal mean blood pressure. The result of this alteration was an immediate and practically complete cessation of urinary secretion.

SUMMARY

Physiological perfusion of organs is a matter of great difficulty. Much of the work done with organ perfusion is of little value because a proper appreciation of the limitations of the method does not obtain among laboratory workers.

The use of physiological saline, Locke's solution or various modified solutions with or without red blood corpuscles does not permit of *physiological perfusion* of organs. The contact of these salt solutions with the tissue cells will result in profound injury or actual cell destruction. Any deduction made from experiments of this nature must be limited by these facts just outlined.

BIBLIOGRAPHY

- (1) KERR, HURWITZ AND WHIPPLE: This Journal, 1918, xlvii, 356, 370, 379.
- (2) GUTHRIE: Arch. Int. Med., 1910, v, 232.
- (3) LE GALLOIS: Experiments on the principle of life (Transl. by N. C. and J. G. Nancrede), Philadelphia, 1813.
- (4) KAY: Journ. des progrès d. sci. et inst. Médic., 1828, x, 67; xi, 18 (cited by Brown-Séquard, see (6)).
- (5) LÖBELL: Diss. Marburg, 1849 (cited by Jacobj, see (11)).
- (6) BROWN-SÉQUARD: Journ. de la Physiol. de l'Homme et des Animaux, 1858, i, 95, 353.
- (7) LUDWIG AND SCHMIDT: Leipziger Ber., 1868, xx, 12.
- (8) VON SCHRÖDER: Arch. exper. Path. u. Pharm., 1882, xv, 364.
- (9) VON FRY AND GRUBER: Arch. Anat. u. Physiol. (Physiol. Abth.), 1885, 519.
- (10) HAMEL: Zeitschr. Biol., 1888, xxv, 474.
- (11) JACOBJ: Arch. exper. Path. u. Pharm., 1890, xxvi, 388.

- (12) JACOB: Arch. exper. Path. u. Pharm., 1895, xxxvi, 330.
- (13) BRODIE: Journ., Physiol., 1903, xxix, 266.
- (14) HOFFMANN: Arch. gesamt. Physiol., 1903, c, 242, 249.
- (15) RICHARDS AND DRINKER: Journ. Pharm. Exper. Therap., 1915, vii, 467.
- (16) FRIEDMANN: Biochem. Zeitschr., 1910, xxvii, 87.
- (17) MANDEL: Zeitschr. f. biol. Technik u. Methodik, 1908, i, 44.
- (18) KINGSBURY: Journ. Biol. Chem., 1916, xxviii, 167.
- (19) HOOKER: This Journal, 1910, xxvii, 24.
- (20) MARTIN: Studies from the Biol. Laby., Johns Hopkins Univ., 1881, ii, 119.
Reprint in Memoirs from the Biol. Laby., Johns Hopkins Univ., 1895, iii, 1.
- (21) BAINBRIDGE AND EVANS: Journ. Physiol., 1914, xlviii, 278.
- (22) HOOKER: This Journal, 1915, xxviii, 200.
- (23) WHIPPLE AND COOKE: Journ. Exper. Med., 1917, xxv, 461.
- (24) WHIPPLE, STONE AND BERNHEIM: Journ. Exper. Med., 1913, xvii, 286.
- (25) MAGNUS: Arch. exper. Path. u. Pharm., xlvii, 200.
- (26) SOLLMANN: This Journal, 1906, xv, 121.
- (27) PRÉVOST AND DUMAS: Ann. de Chimie, 1821, xviii, 294.
- (28) MAGENDIE: Journ. de Physiol., 1822, ii, 338 (cited by Jullien, Transfusion du Sang, 1875).
- (29) MAGENDIE: Leçons sur les Phénomènes Physiques de la Vie, 1838, ii.
- (30) BISCHOFF: Arch. f. Anat. Physiol. u. Wissensch. Med., 1835, 347.
- (31) GOLL: Zeitschr. f. rat. Med., 1854, iv, 78.
- (32) POLLI: Arch. gén. de Med., Oct. and Nov., 1854 (cited by Jullien, Transfusion du Sang, 1875).
- (33) PANUM: Arch. path. Anat. u. Physiol., 1864, xxix, 241.
- (34) PONFICK: Arch. f. Path. Anat. u. Physiol., 1875, lxii, 273.
- (35) STEVENS AND LEE: Studies from the Biol. Laby., Johns Hopkins Univ., iii, 99 (cited by Pfaff and Vejux-Tyrode, see (36)).
- (36) PFAFF AND VEJUX-TYRODE: Arch. f. exper. Path. u. Pharm., 1903, xlix, 324.
- (37) WHIPPLE, SMITH AND BELT: This Journal, 1920, lii, 72.
- (38) WHIPPLE AND GOODPASTURE: This Journal, 1914, xxxiii, 50, 70.
- (39) HOOKER: Journ. Pharm. Exper. Therap., 1913, iv, 443.
- (40) GRUBE: Journ. Physiol., 1903, xxix, 276.
- (41) COOPER: Guy's Hospital Repts., 1836, i, 457.
- (42) LABORDE: Cited by Hayem and Barriere (see (43)).
- (43) HAYEM AND BARRIERE: Arch. de Physiol., 1887, x, 1.
- (44) GUTHRIE, PIKE AND STEWART: This Journal, 1906, xvii, 344.
- (45) MUNK: Arch. path. Anat. u. Physiol., 1887, evii, 291.
- (46) GESELL: This Journal, 1913, xxxii, 70.

BLOOD VOLUME STUDIES

I. EXPERIMENTAL CONTROL OF A DYE BLOOD VOLUME METHOD

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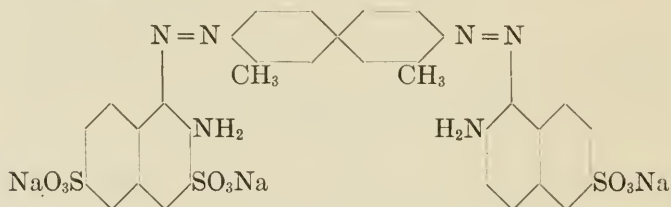
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Studies of blood volume developed in this laboratory as the result of work which was being done in the field of pigment metabolism. Both bile pigment and blood pigment were being studied with particular regard to reciprocal pigment production and destruction in the bile and blood respectively (1). It is obvious that no comprehensive study of blood pigment can be made without reasonably accurate determinations of blood volume. It is remarkable how much effort has been expended in a study of cell counts and hematocrit readings under various experimental conditions with no knowledge concerning the blood volume figures. If a critic chooses to assume any fluctuations in blood volume, all the carefully drawn hypotheses in such papers must become valueless. That such fluctuations in blood volume do occur following a variety of experimental conditions will be shown in experiments to be published in this series of papers.

The final blood volume method as outlined in this paper is the end result of a series of modifications made in the original dye method published by Keith, Rowntree and Geraghty (2). Vital red, the dye used in their experiments, was not obtainable in sufficient quantity and it was necessary to substitute the dye "brilliant vital red." Dr. Herbert M. Evans, Professor of Anatomy, with his expert knowledge of dyes, when consulted concerning the use of other dyes in the estimation of plasma volume, suggested "brilliant vital red." This opportunity is taken to express appreciation of Doctor Evans' interest in

this work and the valuable assistance and materials which he has given so generously.

"Brilliant vital red" is tolidine combined with one molecule of β naphthylamine, 3,6 disulfonic acid and one molecule of β naphthylamine 6 monosulfonic acid.



It is readily soluble in water and does not dialyze against water through celloidin sacs. It is harmless when injected intravenously in the amounts used for the estimation of plasma volume. In none of the many experiments with the blood volume method which have been performed by us have any symptoms been noted that could be attributed to toxicity on the part of the dye. To further test this point 20 cc. of a 1 per cent solution of brilliant vital red were injected into the jugular vein of a mongrel bull pup weighing 6.6 kilos. Although this is approximately fifteen times the amount injected in a blood volume determination no evil effects were observed except a rise of 0.7°C . in temperature for a period of two hours. There is no evidence that the dye has any hemolytic action on normal dog's blood.

METHOD

Normal dogs were used in all these experiments. A hypodermic needle is inserted into the vein and 10 cc. of blood drawn (with as little compression of the vein as possible) into a dry well-vaselined syringe. The blood is immediately placed in a 15 cc. graduated hematocrit tube containing 2 cc. of a 1.6 per cent solution of sodium oxalate. The blood and oxalate are mixed by inversion and the tube stoppered. A standard amount of the dye is drawn up into a syringe along with 5 to 10 cc. of 0.9 per cent saline. This dye solution is now injected into the jugular vein, the dye being washed completely out of the syringe into the blood stream by means of a few cubic centimeters of saline. The dye solution is 1 per cent strength and is given in the amount of 1 cc. per 5 kilos body weight. This is the amount arbitrarily taken by us but more or less may be used to suit individual taste in color readings.

Exactly four minutes after the injection of this dye a clean needle is again inserted into the jugular vein (preferably of the opposite side) and another 10 cc. sample of blood drawn and placed in another hematocrit tube also containing 2 cc. of 1.6 per cent sodium oxalate. The hematocrit tubes are now centrifugalized at 2500 revolutions a minute for 30 minutes. The total contents of the tube and the number of cubic centimeters of blood cells are now noted. Two cubic centimeters of the dye-colored plasma are pipetted off and diluted in a small tube with 4 cc. of 0.9 per cent sodium chloride. This unknown is then read in a colorimeter against a standard prepared as follows:

1. Seventy-five-hundredths cubic centimeters of 1 per cent brilliant red vital is pipetted into a 200 cc. volumetric flask which is then made up to the mark with distilled water.

2. Five cubic centimeters of this aqueous dye solution are then diluted with 5 cc. of clear dye-free plasma (obtained from the first sample of blood drawn from the animal) and 5 cc. of 0.9 per cent sodium chloride, making in all 15 cc. of standard dye solution.

Against this standard the above unknown is read and expressed in per cent. This value will henceforth be referred to as R .

Let us assign the following values to be used in the formula below:

D equals the number of cubic centimeters of 1 per cent brilliant vital red injected into the animal.

C , the correction for the dilution by oxalate present equals the total number of cubic centimeters of oxalated plasma present in the second sample of blood drawn, minus 2 divided by the total number of cubic centimeters of oxalated plasma present in the same tube. This value expresses the ratio between the actual concentration of dye in the plasma when diluted with oxalate solution to the value when not so diluted.

R equals the observed colorimetric reading (in per cent of the standard).

Plasma per cent means the percentage of the whole blood which the plasma constitutes, and is obtained by dividing the total number of cubic centimeters of oxalated plasma present in the hematocrit tube minus 2, by the total contents of the tube in cubic centimeters minus 2.

Then

$$\text{The plasma volume (in cc.)} = \frac{26666.67 D C}{R}$$

$$\text{The blood volume} = \frac{\text{Plasma Volume} \times 100}{\text{Plasma Per Cent}}$$

The above formula for plasma volume may be derived as follows: The standard for color comparison contains 0.75 cc. of 1 per cent dye in 200 cc. of fluid, or 1 cc. in 266.66+ cc. D cc. of 1 per cent dye (the amount injected) will impart the same color intensity to 266.6667 D cc. of fluid.

If, however, the color intensity is $\frac{R}{100 C}$, (i.e., the colorimetric reading in per cent corrected for the dilution of the plasma by the oxalate solution), the number of cubic centimeters of fluids equals $\frac{(266.6667D)(100C)}{R}$
 or $\frac{26666.67 D C}{R}$

METHOD CONTROLS

In the method as outlined above the blood drawn from the animal is mixed with a known amount of an isotonic solution of sodium oxalate (see below) instead of with solid sodium oxalate as was done by Keith, Rowntree and Geraghty (2). The experiments cited in table 1 illustrate the fact that shrinking of blood cells results when even small amounts of solid sodium oxalate are added. Thus the minimal amount of oxalate which can be used (10 mgm. to 10 cc. of blood) causes on an average a shrinkage of about 3 per cent in the cell volume. In actual practice a considerable excess of oxalate may be added unless carefully weighed out. In such cases the error is still larger and may in fact be several times this size.

To obtain the true hematocrit value the anticoagulant used must be isotonic with blood. It is true that samples of blood drawn from different individuals differ from each other in tonicity, but the variation is very slight except in certain pathological conditions such as uremia. The tonicity of normal blood is generally stated to be equal to that of a 0.95 per cent solution of sodium chloride. As far as we have been able to learn, no accurate physical-chemical investigations have been made to determine the concentration of sodium oxalate isotonic with physiological sodium chloride. Assuming that the dissociations of sodium and potassium oxalates are not very far different we find by calculation from the tables of Noyes and Johnston (3) that a 1.6 or 1.7 per cent solution of sodium oxalate would be approximately isotonic with a 0.95 per cent solution of sodium chloride. Valuable advice covering this physical-chemical work was kindly rendered us by Dr. Carl L. A. Schmidt of the Department of Biochemistry.

A number of experiments have been carried out to determine by *biological tests* that concentration of sodium oxalate which, placed in contact with normal red blood cells, produces neither shrinking nor swelling of these cells. Table 2 below shows the results of two experiments on defibrinated normal dog's blood. In both cases the 1.6 per cent solution of sodium oxalate is approximately isotonic. The effect of oxalate solutions on the blood cells of peptonized blood is given in the third column of the same table. 1.4 per cent is obviously hypotonic, while 1.7 per cent is hypertonic. By interpolation the point of isotonicity is found to be about 1.5 per cent. We may, then, from the physical-chemical data supported by biological experiments, conclude that 1.6 per cent sodium oxalate is approximately isotonic with normal dog's blood.

Experiment 222. Tonicity of solutions of sodium oxalate

Two hundred and thirty cubic centimeters of blood were rapidly aspirated from the jugular vein of a normal 40-pound dog (17-160). This blood was immediately defibrinated by whipping.

Two cubic centimeters of sodium oxalate solutions of varying concentrations were pipetted into each of a series of seven tubes. The eighth was left empty. A second and identical series was set up as duplicates. Into each of these tubes were pipetted 9 to 10 cc. of the well-mixed defibrinated blood. The contents of the tubes were mixed, the tubes corked and centrifugalized for one-half hour at 3000 revolutions per minute. The quantity of packed cells and of supernatant fluid was read off on each of the tubes. After making proper allowance for the quantity of fluid added along with the oxalate, the per cent of cells was calculated. The averages for the duplicates are given in table 2.

Identically the same procedure was carried out on a similar quantity of blood obtained from another normal 42-pound dog (621). The results are given also in table 2.

Experiment 224. Tonicity of solutions of sodium oxalate

Three grams of "Witte's Peptone" dissolved in 20 cc. of Locke's solution were injected rapidly into the jugular vein of a normal young 10-pound female terrier under complete ether anesthesia. After 30 minutes a cannula was placed in the carotid artery and samples of blood were drawn into each of a series of four hematocrit tubes. Of these tubes three contained each 2 cc. of solutions of sodium oxalate of varying concentration. The fourth tube contained no oxalate and was prevented from clotting by the familiar "peptone reaction." This experiment supplements the data furnished in the two defibrination experiments. All of the tubes were then centrifugalized simultaneously for 30 minutes at 3000 revolutions per minute. The quantity of packed cells and of supernatant fluid was read off on each of the tubes. After making proper allowance for the quantity of fluid added along with the oxalate, the per cent of cells was calculated. The results are given in table 2.

Dye removed by blood coagulation. When the error due to the use of dry powdered oxalate was first appreciated we turned to the use of blood serum obtained by coagulation in a vaselined tube. The blood was obtained as usual four minutes after dye injection, allowed to clot

TABLE 1

Red blood cell hematocrit modified by solid sodium oxalate

DOG	QUANTITY OF DEFIBRI- NATED BLOOD	HEMATOCRIT (IN PER CENT RED BLOOD CELLS) ON ADDING SOLID SODIUM OXALATE						
		0 mgm.	10 mgm.	20 mgm.	30 mgm.	50 mgm.	70 mgm.	100 mgm.
	cc.							
19-128	10	57.9	55.0	52.0	49.5	43.2	45.6	42.7
17-160	10	57.5	54.5	53.0	50.0	49.5	46.2	44.0
19-106	10	49.8	46.7	45.7	43.8	44.3	43.4	40.0

A fair approximation of the usual amount of dry powdered oxalate used in routine work may be placed at 40 to 60 mgm. per 10 cc. blood.

TABLE 2

Tonicity of solutions of sodium oxalate

PER CENT SODIUM OXALATE USED	HEMATOCRIT BLOOD CELL PER CENT		
	Experiment 222, dog 17-160	Experiment 222, dog 621	Experiment 224, dog 19-107, young adult
1.0	48.0	63.0	63.7
1.1	47.7	62.0	
1.2	47.4	61.3	
1.3	46.6	61.2	
1.4	46.1	59.9	61.1
1.5	46.3	60.4	
1.6	45.2	59.1	
Control with no oxalate	45.1	58.9	59.6
1.7			56.6
	Defibrination of blood	Defibrination of blood	Peptone shock blood incoagu- lable

and the serum collected after centrifugalization. This serum was then compared colorimetrically with a known dye standard. Such steps would simplify the procedure as it would then be unnecessary to allow for dilution by the oxalate solution.

This method has been employed as a routine procedure in a large number of experiments which have been performed in this laboratory. Experiments cited below show that certain dangers are involved. Thus while experimenting with a recently purchased sample of brilliant vital red obtained from an English manufacturer it was found that in the process of clotting a certain amount of dye was removed from solution. This is shown in experiment 219. On an average in the three cases here cited about 7 per cent of the dye was removed from the plasma during the process of clotting. For this reason the blood volume values are too high when the serum is used for the dye reading.

The stock dye which has been used in the previous work was tested in a similar series of experiments by Mrs. F. S. Robscheit in this laboratory. In the case of this dye apparently none of the dye was so removed. In several tests made with this same dye we have confirmed this observation. The routine blood volume work already done in this laboratory with this serum method was all done with the old stock dye and therefore cannot be criticised from this standpoint. It is noteworthy that the dye obtained from the English manufacturer was of a different shade of red from the samples which we had previously used. Furthermore it was decidedly weaker in strength so that it was necessary to use a 2 per cent solution for injection instead of the customary 1 per cent solution.

Whether all samples of brilliant vital red when present in larger amounts (as in some of the experiments cited in the following paper) would be removed in the process of clotting is not as yet known. In view of this uncertainty and in view also of the differences in dyes obtained from different manufacturers we feel that to avoid possible error all samples should be collected in isotonic oxalate solution in the manner already described.

Experiment 219. The effect of blood clotting on the concentration of dye in the plasma

Three healthy young adult dogs were used in the following experiment:

Dog 19-38. Female Coach dog. Weight 24.68 pounds.

Dog 19-39. Female mongrel terrier. Weight 24.25 pounds.

Dog 18-92. Male mongrel terrier. Weight 18.75 pounds.

One cubic centimeter of brilliant vital red (English) for each five kilos body weight was injected intravenously. The blood volume determination was carried out in the usual way with the exception that in addition to the ordinary hematocrit sample taken four minutes after the injection of the dye, a 10 cc. sample was also drawn at this time into a dry vaseline-lined heavy-walled test tube and allowed to stand 30 minutes. At the end of this time the clot was

freed from the wall of the tube with a piece of wire. After centrifugalization (30 minutes) the serum was drawn off and diluted with two volumes of 0.9 per cent sodium chloride solution. The dye-colored oxalated plasma was obtained from the hematocrit tube in the same way and was similarly diluted. Both of these diluted dye-containing samples were then read colorimetrically against a standard prepared as follows: 0.5 cc. of the 2 per cent brilliant vital red was accurately brought up to 100 cc. in a volumetric flask; 5 cc. of this mixture were mixed with 5 cc. of dye-free plasma and 5 cc. of 0.9 per cent sodium chloride.

The observed readings and estimations for plasma volume and blood volume for each of the three dogs are given in table 3 below. It will be observed that the samples were read against a standard which is stronger than usual. This must be taken into account in making the plasma volume and blood volume calculations.

TABLE 3

Experiment 219. The effect of blood clotting on the concentration of dye in the plasma

SPECIMEN READ AGAINST STANDARD	OBSERVED READ- ING AGAINST STANDARD			ESTIMATED PLASMA VOLUME			HEMATOCRIT (CELLS)			ESTIMATED BLOOD VOLUME		
	Dog 19-38	Dog 19-39	Dog 18-92	Dog 19-38	Dog 19-39	Dog 18-92	Dog 19-38	Dog 19-39	Dog 18-92	Dog 19-38	Dog 19-39	Dog 18-92
	per cent	per cent	per cent	cc.	cc.	cc.	per cent	per cent	per cent	cc.	cc.	cc.
Serum*.....	92	83	64	487	530	533	(52.1)	(60.5)	(48.2)	1017	1342	1029
Oxalated plasma (corrected for the dilution by the oxalate so- lution)	99	91	70	453	485	487	52.1	60.5	48.2	946	1228	940

*This indicates that blood coagulation removed a definite per cent of this dye from the plasma. For this reason the blood volume values are too high when the serum is used for the dye readings.

Centrifugalization factors. In order to determine the minimum length of time that is required to cause by centrifugalization at 2500 revolutions a minute a completion of the process of sedimentation, a number of 15 cc. hematocrit tubes filled with oxalated dog's blood were centrifugalized varying lengths of time. The distance of the bottom of the tube from the center of the circle of rotation equals 27 cm. Twenty minutes were found to be insufficient; however, the packing at the end of thirty minutes was in most cases as complete as at any subsequent time. According to Köppe (4) complete packing of the cells is accompanied by a translucency of the layer of packed cells. Such translucency according to him occurs only if the tubes are centrifugalized at a speed

of not less than 5000 revolutions a minute. The experiments no. 253 and 268 presented below show that if any of the dye-colored plasma remains in the interstices it is so small in amount as to be negligible. Moreover, we have performed experiments which show that packed cells from dye-containing blood when washed with very small amounts of isotonic saline impart to this saline only an exceedingly small trace of dye. For all practical purposes therefore the packing may be considered complete at the end of thirty minutes.

Experiment 268. The principle of the blood volume method tested in vitro

A total of 1500 cc. of blood was drawn from the jugular veins of four normal dogs into 75 cc. of 1.6 per cent sodium oxalate. This blood was thoroughly mixed. Nine hundred cubic centimeters were poured into a dry 1000 cc. volumetric flask. Two cubic centimeters of 1 per cent brilliant vital red were added and the flask made up to mark with oxalated blood. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time 14 cc. were quickly pipetted into a dry 15 cc. graduated hematocrit tube. This tube of blood was centrifugalized for 30 minutes at 3000 revolutions per minute. The quantity of packed cells and of supernatant fluid was then read off and the per cent of blood cells calculated to be 53.5 per cent.

Two cubic centimeters of the dye-tinged supernatant fluid were mixed with 4 cc. of 0.9 per cent NaCl and read against a standard prepared as follows: 0.75 cc. of 1 per cent brilliant vital red was accurately brought up to 200 cc. with distilled water in a volumetric flask. Five cubic centimeters of this were mixed with 5 cc. of the clear dye-free oxalated plasma and 5 cc. of the 0.9 per cent saline. Against this standard the above diluted sample of dye-colored plasma reads 117.3 per cent.

With this concentration of dye the number of cubic centimeters of oxalated plasma in the total 1000 cc. of blood would, from the previously given formula, be $\frac{26666.67 \times 2}{117.3} = 455$. The actual amount of oxalated plasma present (as indicated by the hematocrit) was 465 cc.

Experiment 253. Blood volume in vitro

Three hundred cubic centimeters of dog's blood were drawn into a bottle containing 35 cc. of 1.6 per cent sodium oxalate. This blood was thoroughly mixed. Two hundred cubic centimeters were poured into a dry 250 cc. volumetric flask, 0.455 cc. of 1 per cent brilliant vital red was added and the flask made up to mark with oxalated blood. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time two graduated 15 cc. centrifuge tubes were filled, allowed to stand for several minutes and centrifugalized at 2500 revolutions a minute for 30 minutes. The quantity of packed cells and of supernatant fluid was then read off and the per cent of blood cells calculated to be 53.9 per cent.

Two cubic centimeters of the dye-tinged supernatant fluid were mixed with 4 cc. of 0.9 per cent NaCl and read against a standard prepared as follows: 0.75 cc. of 1 per cent brilliant vital red was accurately brought up to 200 cc. with distilled water in a volumetric flask. Five cubic centimeters of this were mixed with 5 cc. of the clear dye-free oxalated plasma (obtained by centrifugalization) and 5 cc. of the 0.9 per cent NaCl. Against this standard the above diluted sample of dye-colored plasma read 100 per cent.

With this concentration of dye the number of cubic centimeters of oxalated plasma in the total 250 cc. of oxalated blood would, from the previously given formula, be $\frac{26666.67 \times 0.455}{100} = 121$. The actual amount present, as indicated by the hematocrit, was 115 cc.

Blood volume method tested in vitro. The dye method for the determination of blood volume has been tested *in vitro* on large quantities of oxalated

TABLE 4
Rate of elimination of brilliant vital red from circulation

BLOOD FROM DOG NUMBER	ACTUALLY OBSERVED COLORIMETRIC READING OF THE TWO MINUTE SAMPLE CORRECTED FOR OXALATE	RELATIVE CONCENTRATION OF DYE IN PLASMA AFTER INJECTION OF DYE (TWO MINUTE SAMPLE TAKEN AS 100)				
		Two min- utes after injection	Four min- utes after injection	Twenty min- utes after injection	Three hours after injection	Twenty-four hours after injection
18-39	103.6	100	99.2	93.9		10 to 15
18-28	122.2	100	99.3	89.0	62.4	10 to 15
19-123	136.0	100	102.6	93.0	64.0	10 to 15
19-84	138.3	100	98.3	91.1	59.8	10 to 15
Average..	125.0	100	99.9	91.8	62.1	10 to 15

dog's blood in the experiments given above (268 and 253). The theoretical and the estimated quantity of oxalated plasma in the flask in one case differ by about 5 per cent; in the other by less than 2 per cent. The dye therefore is not absorbed to any appreciable extent by the blood cells nor is it to be found to any appreciable extent in the interstices between the blood cells when the latter are packed by the degree of centrifugalization employed. Furthermore, experiments have shown that no appreciable amount of dye could be extracted from this layer of packed cells by washing with small amounts of physiological saline.

Elimination of the dye from circulation. Any blood volume method based on the dilution of a substance introduced into the blood stream depends for its accuracy on a slow rate of elimination from circulation. The rate of elimination of brilliant vital red is shown in table 4. In this table are summarized experiments on four normal active young adult

dogs. An amount of dye equal to that customarily used in blood volume estimation was injected into each dog in the customary way. Ten cubic centimeter samples were collected at intervals after the injection of the dye. As can be seen from table 4, the concentration of the dye in the plasma in most cases is the same at the end of two minutes as at

TABLE 5

EXPERI- MENT NUMBER	SEX	DESCRIPTION	WEIGHT	BLOOD VOLUME	PLASMA VOLUME	TOTAL BLOOD CELL VOLUME	HEMATO- CRIT CELLS	BLOOD PER 100 GRAM BODY WEIGHT
			<i>kgm.</i>				<i>per cent</i>	<i>cc.</i>
305			7.27	640	310	330	51.6	8.80
233	f	Fox	8.52	892	365	527	59.1	10.47
237	m	Fox	9.09	1041	478	563	54.1	11.45
230	f	Coach	10.97	1197	601	596	49.8	10.91
235	f	Mongrel Spitz	11.14	1255	557	698	55.6	11.27
251	m	Mongrel	13.18	1357	643	714	52.6	10.30
257	m	Mongrel	13.18	1293	742	551	42.6	9.81
258	m	Spitz	13.18	1103	502	601	54.5	8.37
234		Mongrel	13.64	1439	525	914	63.5	10.55
261	m	Mongrel	13.64	1225	767	458	37.8	8.98
256	m	Mongrel	13.86	1314	644	670	51.0	9.48
211	m	Setter	15.91	1835	936	899	49.0	11.53
262	m	Bull	16.36	1487	769	718	48.3	9.09
240	f	Bull-terrier	17.73	1867	788	1079	57.8	10.53
241	m	Bull	17.73	1730	865	865	50.0	9.76
249	m	Setter	17.73	1867	958	909	48.7	10.53
247	m	Spaniel	18.41	2080	1029	1051	50.5	11.30
231	m	Shepherd	18.73	1614	1069	545	34.4	8.62
254	m	Shepherd	20.00	1955	919	1036	53.0	9.78
299			20.00	2119	945	1174	55.4	10.60
236	f	Terrier	20.57	2264	840	1424	62.9	11.01
238	m	Mongrel	22.05	2166	1025	1141	53.6	9.82
Average of dogs weighing from 7 to 15 kgm.			11.61	1160	558	602	52.0	10.04
Average of dogs weighing from 15 to 23 kgm.			18.65	1908	922	986	51.2	10.23
Average of all dogs			15.13	1538	740	794	51.6	10.13

the end of four minutes. At the end of twenty minutes, however, about 6 to 11 per cent is lost while in twenty-four hours only about 10 per cent remains. Blood volume estimations based on samples collected at the four-minute interval cannot therefore be invalidated because of the elimination of dye from circulation. It seems probable that the mixing of dye with the plasma is complete at the end of four minutes.

The blood volume of normal dogs. Blood volume figures for twenty-two normal dogs, young and adult, maintained on the usual mixed diet are given in table 5. The average for all animals shows 10.13 cc. of blood per 100 grams of body weight. This value corresponds fairly closely to the results of Keith; Rowntree and Geraghty (dye method) (2), and to those of Meek and Gasser (acacia method) (5), but is considerably higher than that obtained by Grehand and Quinquaud (6) with the carbon monoxide method (8.2 cc. per 100 grams body weight), and also that obtained by the method originally employed by Welcker, which depends on washing out or extracting all of the blood and esti-

TABLE 6

Female mongrel bull dog, weight 22 pounds. Hemorrhage 262 cc. in 3 minutes

DOG NUMBER	DATE	BLOOD VOL- UME	PLASMA VOL- UME	TOTAL CELL VOLUME	HEMATOCRIT (CELLS)	TOTAL PRO- TEIN	ALBUMIN	GLOBULIN	NON-PROTEIN
16-160	March 4.....	1006	533	473	47.0 <i>per cent</i>				
	March 6 (before hem- orrhage).....					5.7	4.1	1.6	2.5
	March 6 (1 minute after hemorrhage).....					5.6	4.1	1.5	2.5
	March 6 (5 minutes after hemorrhage).....	741	363	378	51.0	5.3	4.1	1.2	2.3
	March 6 (30 minutes after hemorrhage)....								
	March 7.....					4.7	3.5	1.2	2.3
	March 9.....	844	591	253	30.0	4.3	3.3	1.0	2.5
	March 15.....								
						4.9	3.7	1.2	2.5

inating the hemoglobin. By this method about 7.8 cc. of blood per 100 grams body weight was obtained (7). The reasons for the difference between these methods are as yet not entirely clear. This problem is receiving further study in this laboratory.

In this series practically no difference exists in the amount of blood per unit body weight in respect to the size of the animal.

Hemorrhage experiments. In a number of animals the effect of acute hemorrhage on blood and plasma volume was studied. The protocols of four such experiments are presented in tables 6, 7, 8 and 9. In all experiments healthy, sound young dogs were used. The first blood volume estimation in each experiment was performed two days

before the day of the hemorrhage. It is recognized that in individual cases the blood volume may change somewhat in the space of two days. In order to minimize this error the animals were kept under as nearly constant conditions as possible throughout the course of the experiment, all animals being kept on a uniform diet of cracker-meal, lard and butter for a period of five days preceding the hemorrhage. Hemorrhage was effected by rapid withdrawal of blood by aspiration through a needle inserted in the external jugular vein. The animals were but slightly depressed and showed but a slight temporary amount of dyspnea as a result of the loss of blood.

TABLE 7

Black male mongrel pup, weight 20 pounds. Hemorrhage 404 cc. in 3 minutes

DOG NUMBER	DATE	BLOOD VOL- UME	PLASMA VOL- UME	TOTAL CELL VOLUME	HEMATOCRIT CELLS <i>per cent</i>	TOTAL PRO- TEIN	ALBUMIN	GLOBULIN	NON-PROTEIN
16-140	March 4.....	1616	711	905	56	5.0			
	March 6 (before hemor- rhage).....					5.4	3.7	1.7	2.5
	March 6 (1 minute after hemorrhage).....					5.2	4.1	1.1	2.1
	March 6 (5 minutes after hemorrhage).....	1130	531	599	53	5.3	3.9	1.4	2.1
	March 6 (30 minutes after hemorrhage)....					5.1	3.7	1.4	2.1
	March 7.....					4.9	3.6	1.3	2.2
	March 9.....	1222	880	342		4.5	2.9	1.6	2.2
	March 15.....					4.7	3.1	1.6	2.2

The effect of the procedure on the blood volume, plasma volume and cell volume is summarized in table 10. The amount of plasma and of cells removed is calculated from the total amount of blood withdrawn on the assumption that the blood withdrawn contained the same percentage of cells as was present in the blood at the time of performing the first blood volume. Although a certain amount of variation exists in individual experiments, the averages show that the fall in both cell volume and in plasma volume is within a few cubic centimeters of the theoretical. In the course of three days, however, the plasma volume has returned to a level slightly above the original level. Due to the decreased cell volume the blood volume is still

TABLE 8

Female bull coach mongrel, weight 18 pounds. Hemorrhage 244 cc. in 4 minutes

DOG NUMBER	DATE	BLOOD VOL- UME	PLASMA VOL- UME	TOTAL CELL VOLUME	HEMATOCRIT (CELLS)	TOTAL PRO- TEIN	ALBUMIN	GLOBULIN	NON-PRO- TEIN
17-157	March 4.....	976	537	439	<i>per cent</i> 45				
	March 6 (before hemor- rhage).....					5.7	4.3	1.4	2.1
	March 6 (1 minute after hemorrhage).....					5.4	3.7	1.7	2.1
	March 6 (5 minutes after hemorrhage).....	724	427	297	41	5.0	3.5	1.5	1.8
	March 6 (30 minutes after hemorrhage)....				40	4.9	3.5	1.4	1.8
	March 7.....					5.0	3.9	1.1	1.6
	March 9.....	818	597	221	27	4.9	3.9	1.0	1.8
	March 15.....					4.9	3.5	1.4	2.1

TABLE 9

Male mongrel bull dog, weight 27.5 pounds. Hemorrhage 264 cc. in 2 minutes

DOG NUMBER	DATE	BLOOD VOL- UME	PLASMA VOL- UME	TOTAL CELL VOLUME	HEMATOCRIT (CELLS)	TOTAL PRO- TEIN	ALBUMIN	GLOBULIN	NON-PRO- TEIN
17-205	March 4.....	1055	538	517	<i>per cent</i> 49				
	March 6 (before hemor- rhage).....					3.4	3.9	1.5	2.7
	March 6 (1 minute after hemorrhage).....					5.8	4.2	1.6	2.1
	March 6 (5 minutes after hemorrhage).....	757	424	333	44	5.3	4.1	1.2	2.3
	March 6 (30 minutes after hemorrhage)....					5.2	3.9	1.3	2.1
	March 7.....					5.2	3.5	1.7	2.3
	March 9.....	840	605	235	28	4.9	3.5	1.2	2.5
	March 15.....					5.0	3.9	1.1	2.1

below the original figure. The fact that the cell volume is lower at the end of three days than immediately following the hemorrhage is a peculiar and as yet unexplained phenomenon. More data are being collected on this point.

In all of the hemorrhage experiments the serum proteins were estimated by the micro-refractometric method of Robertson (8). A summary of the figures given in detail in the protocols of the individual experiments is presented in table 11. The amount of circulating serum proteins is decreased as a result of hemorrhage by very nearly the theoretical amount. Regeneration, however, has in the course of

TABLE 10

The effect of hemorrhage upon blood volume

DOG NUMBER	WITHDRAWN		BLOOD VOLUME			PLASMA VOLUME			TOTAL CELL VOLUME		
	Of plasma	Of blood cells	Before	Five minutes after	Three days after	Before	Five minutes after	Three days after	Before	Five minutes after	Three days after
	cc.	cc.									
16-160	139	123	1006	741	844	533	363	591	473	378	253
16-140	142	262	1616	1130	1222	711	531	880	905	599	342
17-157	134	110	976	724	818	537	427	597	439	297	221
17-205	135	129	1055	757	840	538	424	605	517	333	235
Average.....	138	156	1164	838	931	580	436	668	583	402	263

TABLE 11

The effect of hemorrhage upon the circulating serum proteins

DOG NUMBER	SERUM PROTEIN WITHDRAWN	CIRCULATING SERUM PROTEINS			SERUM PROTEIN REGENERATED	
		Before hemorrhage	Five minutes after hemorrhage	Three days after hemorrhage	In five minutes	In three days
	grams	grams	grams	grams	grams	grams
16-160	8.07	30.38	19.24	25.41	-3.07	3.10
16-140	9.74	38.39	28.14	41.36	-0.51	12.71
17-157	7.72	30.61	21.35	29.25	-1.54	6.36
17-205	7.91	29.05	22.47	29.65	+1.33	8.51
Average.....	8.36	32.11	22.80	31.42	-0.95	7.67

three days restored the circulating proteins almost to the original figure. It is noteworthy, however, that the concentration of protein in the serum is still considerably below the original.

In conclusion and on the behalf of the Department of Anatomy and the Hooper Foundation we wish to express our appreciation for invaluable assistance given by Dr. Carl Alsberg, Chief of the Bureau

of Chemistry, Department of Agriculture, Washington, and Dr. H. D. Gibbs, Chemist in charge of the Color Laboratory, Bureau of Chemistry, Department of Agriculture, Washington. These gentlemen supplied practically all the dye used in the first two papers of this series.

SUMMARY

A simple and accurate method of blood volume determination is outlined in detail. Suitable controls of many factors are submitted and the method tested *in vitro*.

The average normal blood volume for young active dogs of medium weight as determined by this method may be stated as 10.1 per cent body weight. Individual differences are considerable (table 5).

After the rapid withdrawal (hemorrhage) of a known amount of blood, this blood volume method performed immediately will show a *calculated blood volume* remaining which is very close to the *estimated blood volume*.

BIBLIOGRAPHY

- (1) HOOPER AND WHIPPLE: Proc. Amer. Physiol. Soc. This Journal, 1918, xlv, 573.
WHIPPLE AND HOOPER: Proc. Amer. Physiol. Soc. This Journal, 1918, xlv, 576.
- (2) KEITH, ROWNTREE AND GERAGHTY: Arch. Int. Med., 1915, xvi, 547.
- (3) NOYES AND JOHNSTON: Journ. Amer. Chem. Soc., 1909, xxxi, 987.
- (4) KOPPE: Arch. f. d. gesamt. Physiol., 1905, cvii, 187.
- (5) MEEK AND GASSER: This Journal, 1918, xlvii, 302.
- (6) GREHAND ET QUINQUAUD: Compt. Rend. de l'Acad. d. Sciences, 1882, xciv, 1450.
- (7) MÜLLER: Arch. f. Anat. u. Physiol. (Physiol. Abteil), 1901, 459.
- (8) ROBERTSON: Journ. Biol. Chem., 1915, xxii, 233.

BLOOD VOLUME STUDIES

II. REPEATED DETERMINATION OF BLOOD VOLUME AT SHORT INTERVALS BY MEANS OF THE DYE METHOD

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A simple method for the estimation of blood volume in the same animal at short intervals is much to be desired. An accurate method for the estimation of blood volume repeatedly at intervals of minutes or hours will at once give data of interest and value. Such data are not at present available. With the ability to estimate blood volume changes during short intervals we are at once able to study the exchange of fluids which may go on in acute shock, or after hemorrhage or induced plethora. Under such conditions one suspects that there may be exchange of fluid between the tissues and the circulating blood but there are no published experiments which are not open to criticism.

Certain observations of this nature have been reported by a few of the earlier workers—Keith, Rowntree and Geraghty (1), Robertson and Bock (2) and Lamson (3), but no details are given as to the exact method used and only one or two observations are recorded in each report. For this reason and because of our earlier experiments we conclude that these observations were not entirely satisfactory.

When one attempts to repeat the blood volume estimation on the same animal at short intervals he is at once confronted by the fact that after one such determination the plasma remains colored for a period varying from several hours to several days. The presence of dye in the plasma complicates all subsequent blood volume determinations. In order to determine the blood volume a second time under such circumstances, one may again inject intravenously the customary amount of dye and estimate colorimetrically the *increase* in dye concentration in the plasma as a result of such injection. Theoretically this increase in color intensity might be estimated by subtracting the colorimetric reading of a sample of plasma drawn before injection of the

dye from the colorimetric reading of another sample taken after such injection. The increase in color intensity thus determined is, however, subject to the error of two colorimetric readings, the sum of which may in some cases be considerable. For example, the second determination may be made after an interval of a few hours. At such time the amount of dye in the plasma may be small and therefore difficult to estimate accurately because of the normal yellow plasma tint. The procedure used to minimize this error is given below. The mathematical soundness of this procedure will be shown.

METHOD

For a repeated blood volume estimation the dye is injected and the samples collected in the same way as was outlined in the preceding paper (4). Part of this may for convenience be repeated. A hypodermic needle is inserted into the jugular vein and 10 cc. of blood drawn (with as little compression of the vein as possible) into a dry well-vaselined syringe. This blood is immediately run into a 15 cc. graduated hematocrit tube containing 2 cc. of a 1.6 per cent solution of sodium oxalate. The blood and oxalate are mixed by inversion and the tube stoppered. An amount of brilliant vital red (1 per cent) equal in cubic centimeters to the weight of the animal in pounds divided by 11 (i.e., 1 cc. per 5 kilos body weight) is drawn up into a syringe along with 5 to 10 cc. of 0.9 per cent saline. This dye solution is now injected into the jugular vein, the dye being washed completely out of the syringe into the blood stream by means of a few cubic centimeters of normal saline. Four minutes after the injection of dye a clean needle is again inserted into the jugular vein (preferably of the opposite side) and another 10 cc. sample of blood is drawn and placed in another 15 cc. hematocrit tube also containing 2 cc. of 1.6 per cent sodium oxalate.

The second blood volume determination may be done at such time as may be desired—for example, after a period of 10 or 20 minutes. The procedure is exactly similar to that already described and the time interval of 4 minutes is the same as before, that is, 4 minutes from time of injection of the second dye sample to the time of removal of the second dye plasma sample.

The hematocrit tubes are now centrifugalized at 2500 revolutions a minute for 30 minutes. The total contents of the tube and the number of cubic centimeters of blood cells are now noted. Colorimetric readings are then made and the plasma volume estimated as follows:

Calculation of the second blood volume

Let D = the number of cubic centimeters of 1 per cent dye injected into the animal.

C_1 = the total number of cubic centimeters of oxalated plasma present in the first sample of blood drawn minus 2, divided by the total number of cubic centimeters of oxalated plasma present in the same tube. This value expresses the ratio of the actual concentration of dye in the plasma when diluted with oxalate solution, to the value when not so diluted.

C_2 = the total number of cubic centimeters of oxalated plasma present in the second sample of blood drawn minus 2, divided by the total number of cubic centimeters of oxalated plasma present in the same tube. The significance of this figure is the same as that described for C_1 above.

R_1 = the observed colorimetric reading of a sample of dye-tinged plasma (diluted with two parts of saline) taken immediately before the injection of the dye for the second blood volume estimation read against a standard prepared as follows: Of a dye solution containing 0.75 cc. of 1 per cent dye diluted to 200 cc. with water take 5 cc. To this amount add 5 cc. of 0.9 per cent saline and 5 cc. of normal dye-free plasma.

R_2 = the observed colorimetric reading (expressed in per cent) of a sample of dye-colored plasma (diluted with two parts of saline) taken 4 minutes after the injection of the dye for the repeated blood volume estimation, against a standard prepared as follows: 5 cc. of a dye solution containing 0.75 cc. of 1 per cent dye in 200 cc. of water, + 5 cc. of 0.9 per cent saline + 5 cc. of the dye-tinged plasma taken immediately before the injection of dye for the repeated blood volume estimation.

Then:

$$\text{The plasma volume} = \frac{2666666 D C_1 C_2}{C_1 R_2 (R_1 + 100) - 100 C_2 R_1}$$

$$\text{And the blood volume} = \frac{\text{Plasma Volume} \times 100}{\text{Per Cent Plasma}}$$

The total blood-cell volume = blood volume minus plasma volume.

The method above described is essentially the same method as is employed in the estimation of the first blood volume. It will be recalled that in the method as outlined in the previous paper the standard against which the unknown (diluted with two parts of saline) was read, was made up of equal parts of saline, standard dye solution (0.75 cc.

of 1 per cent dye diluted with water to 200 cc.) and normal plasma taken from the same animal immediately before injection of the dye. In performing the second blood volume the unknown and standard are prepared in exactly the same way. In this case, however, the plasma taken immediately before the injection of dye is itself dye-colored (from dye remaining from the previous blood volume determination). This amount of dye (R_1) is present in both standard and unknown.

Hence: $\frac{R_1 + R_3}{R_1 + 100} = \frac{R_2}{100}$ whence R_1 = the colorimetric reading of the plasma before the injection of dye against a standard prepared in the customary way for the first blood volume estimation (equal parts of normal plasma, saline and standard dye solution). R_3 represents the increase in concentration of dye in the plasma as a result of the injection of dye. This formula simply means that the unknown ($R_1 + R_3$) divided by the standard ($R_1 + 100$) equals the observed colorimetric reading expressed in parts per 100 (i.e. $\frac{R_2}{100}$).

The soundness of this last formula is illustrated experimentally in experiment no. 243. In this experiment the standard employed in no. 4 may be taken as 100. The standard used in no. 5 then equals $100 + R_1$ where R_1 (according to no. 4) equals 75. The unknown in no. 5 also contains R_1 and hence may be represented as $R_1 + R_3$ where R_3 equals the increase in dye concentration to be determined. R_2 , the observed reading, is 86. By substituting in the formula $\frac{R_1 + R_3}{R_1 + 100} = \frac{R_2}{100}$ one

obtains $\frac{75 + R_3}{75 + 100} = \frac{86}{100}$ Solving, $R_3 = 75.5$.

It can readily be seen from no. 3 that the increase (R_3) in dye concentration over the residual is 75. The increase as determined colorimetrically with the use of the formula differs from the correct value, therefore, by only 0.5 per cent.

Experiment 243. Measurement of the color increase resulting from the addition of dye to dye-tinged water.

1. Seventy-five one hundredths cubic centimeter of 2 per cent brilliant vital red are accurately diluted with water to 200 cc. in a volumetric flask.

2. Six hundred and seventy-five thousandths cubic centimeter of a 2 per cent solution of the same dye is similarly diluted with water to 240 cc. This is equivalent to diluting 0.56 cc. of dye to 200 cc.

3. Fifty-six hundredths cubic centimeter more of 2 per cent dye is accurately diluted with no. 2 to 200 cc. in a volumetric flask.

4. A standard is prepared as follows: 5 cc. of no. 1 + 10 cc. of water. Against this standard 5 cc. of no. 2 diluted with 10 cc. of water reads 75 per cent.

5. A second standard is prepared as follows: 5 cc. of no. 1 + 5 cc. of no. 2 + 5 cc. of water. Against this standard 5 cc. of no. 3 diluted with 10 cc. of water reads 86 per cent.

It was stated above that mathematically it would be possible to determine the increase in color intensity by a simple subtraction of the residual dye concentration (R_1) from the total dye concentration after addition of more dye. With such procedure it was pointed out that the error in the estimated increase is the algebraic sum of the errors of these two colorimetric readings. Computation shows that by the procedure outlined with the use of the above formula the error in the estimated increase (R_3) is not so great as this algebraic sum provided that R_1 is less than R_3 . Moreover as R_1 becomes smaller its accurate determination becomes increasingly difficult particularly since the interference due to the normal pigments of the plasma becomes more marked. Fortunately an error of considerable magnitude may be made in the estimation of R_1 without seriously affecting the value of R_3 . For example if through error in colorimetric reading R_1 in experiment no. 243 had been incorrectly read as 70 instead of 75, R_3 would have been calculated to be 76.2 instead of 75.5—an error of less than 1 per cent.

In all blood volume determinations the plasma is diluted by the oxalate solution which must necessarily be added to prevent coagulation. Correction must therefore be made for such dilution. The formula

$$\frac{R_1 + R_3}{R_1 + 100} = \frac{R_2}{100} \text{ becomes}$$

$$\frac{\frac{R_1 C_2}{C_1} + R_3}{R_1 + 100} = \frac{R_2}{100} \text{ or } R_3 = \frac{R_1 R_2 C_1 + 100 C_1 R_2 - 100 R_1 C_2}{100 C_1}$$

In the previous paper it was found that Plasma Volume = $\frac{26666.666 D C_2}{R}$. Since R in this formula corresponds to the R_3 in the

formula derived above (R_3 being the increase in dye concentration without correction for dilution by oxalate), R_3 may be substituted in the formula for plasma volume with the following result:

$$\text{Plasma Volume} = \frac{2666667 D C_1 C_2}{C_1 R_2 (R_1 + 100) - 100 C_2 R_1}$$

The experiment cited below (from exper. 218) illustrates the procedure.

Dog, weight 47.5 pounds. At 10.45 a.m. a simple blood volume estimation was carried out. Preparations were then made to repeat the determination as has been outlined above. At 11.07 a.m. a sample of blood drawn from the jugular vein was placed in a 15 cc. hematocrit tube containing 2 cc. of 1.6 per cent sodium oxalate. After centrifugalization this tube was found to contain 5.95 cc. of blood cells. Its total content was 14.75 cc. C_1 estimated as described above is $\frac{68}{88}$. At 11.08 a.m. brilliant vital red (4.30 cc. of 1 per cent solution) together with about 6 cc. of 0.9 per cent sodium chloride were injected into the jugular vein. At 11.12 a.m. another sample of blood was drawn into another hematocrit tube containing also 2 cc. of 1.6 per cent sodium oxalate. After centrifugalization this tube was found to contain 5.6 cc. of blood cells. Its total content was 14.6 cc. C_2 therefore is $\frac{70}{90}$.

One cubic centimeter of plasma from the blood sample drawn at 11.07 a.m. was mixed in a small tube with 2 cc. of 0.9 per cent sodium chloride. This solution was read against a standard prepared as follows: 5 cc. of a dye solution containing 0.75 cc. of 1 per cent brilliant vital red diluted to 200 cc. with water + 5 cc. of 0.9 per cent sodium chloride + 5 cc. of normal plasma (obtained from the same animal at 10.43 a.m., i.e., before he had received the first injection of dye). The test solution reads against this standard 76 per cent. R_1 therefore = 76.

A second standard, this time containing dye-tinged plasma, was prepared as follows: 5 cc. of plasma from the sample of blood drawn at 11.07 a.m. + 5 cc. of a dye solution containing 0.75 cc. of 1 per cent brilliant vital red diluted to 200 cc. with water + 5 cc. of 0.9 per cent sodium chloride. A test solution was prepared by diluting 1 cc. of plasma obtained from the sample of blood drawn at 11.12 a.m. with 2 cc. of 0.9 per cent saline. This solution against the above standard reads 90 per cent. R_2 therefore = 90.

Substituting these values in the formula:

$$\text{Plasma Volume} = \frac{2666667 D C_1 C_2}{C_1 R_2 (R_1 + 100) - 100 C_2 R_1}$$

we have

$$\text{Plasma Volume} = \frac{(2666667) (4.30) \left(\frac{68}{88}\right) \left(\frac{70}{90}\right)}{\left(\frac{68}{88}\right) (90) (76 + 100) - (100) \left(\frac{70}{90}\right) (76)} = 1089 \text{ cc.}$$

The per cent plasma (obtained from an average of the two hematocrit tubes) is 54.4. Since the blood volume = $\frac{\text{Plasma Volume} \times 100}{\text{Plasma Per Cent}}$ the blood volume = $\frac{1089 \times 100}{54.4} = 2002$.

By difference the total cell volume is 913 cc.

DISCUSSION

In tables 1 and 2 the dye method for the repeated estimation of blood volume is tested out *in vitro* on large quantities of oxalated dog's blood. In the experiment cited in table 1 three separate blood volume determinations are made on known amounts of the same sample of oxalated blood. In no case was the error greater than 3.1 per cent while the average error for the three determinations was 2.5 per cent. In the experiment cited in table 2 the errors in the estimation by the dye method were 2.5 and 5.0 per cent respectively in each of two determinations. The mechanical errors involved in the repeated estimations of blood volume by the dye method under such experimental conditions do not exceed 5 per cent.

In the three experiments, tables 3, 4 and 5, repeated blood volume determinations have been made on the same dogs at short intervals of time. In all cases repeated determinations on the same animal give similar results. In no case does any blood volume determination differ from the average of all determinations made on the same animal by more than 5.0 per cent.

We wish to emphasize, however, that these figures apply to determinations completed within two to three hour periods. When longer intervals elapse between blood volumes we may observe fluctuations in blood cell and plasma figures which we cannot at present explain. We feel that sufficient data are submitted to show that these fluctuations are not due to errors inherent in this proposed method. When we consider the various physiological reactions of the blood vessels in response to familiar digestive stimuli we need not be surprised at fluctuations in circulating blood volume. These physiological reactions are merely suggested as a type of the many changes which might modify the fluid or cells circulation in the body at any given time.

Experiment 268. The repeated blood volume method carried out in vitro on oxalated blood.

1. Seven hundred and fifty cubic centimeters of blood were drawn from the jugular veins of each of four normal dogs into each of two bottles containing 75 cc. each of 1.6 per cent sodium oxalate. This blood was thoroughly mixed. A graduated 15 cc. hematocrit tube was filled.

2. Nine hundred cubic centimeters of no. 1 were poured into a dry 1000 cc. volumetric flask. Two cubic centimeters of 1 per cent brilliant vital red were added and the flask made up to mark with no. 1. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time 14 cc. were quickly pipetted into a dry 15 cc. graduated hematocrit tube.

3. Five hundred cubic centimeters of no. 2 were poured into a volumetric flask calibrated to hold 510 cc. One and two hundredths cubic centimeter of 1 per cent brilliant vital red were added and the flask made up to mark with no. 2. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time a dry graduated 15 cc. hematocrit tube was filled with the mixture.

4. Four hundred and eighty-five cubic centimeters of no. 3 were poured into a volumetric flask calibrated to hold 490 cc. Ninety-eight hundredths cubic centimeter of a 1 per cent brilliant vital red were added and the flask made up to mark with no. 3. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time a dry graduated 15 cc. hematocrit tube was filled with the mixture.

All of the hematocrit tubes were corked and centrifugalized for 30 minutes at 2500 revolutions a minute. The quantity of packed cells and of supernatant fluid in each tube was noted.

The standard solutions and dilutions employed in obtaining the colorimetric readings given below were made in the usual manner except that the aqueous dye solution from which the standards were prepared contained 1 cc. of dye diluted to 200 cc. with water instead of the customary 0.75 dye diluted to 200 cc. with water.

In the first volume estimation (no. 2 above) $R_2 = 88$ per cent. R_1 of course in this case is zero.

In the second volume estimation (no. 3 above) $R_2 = 91$ per cent. From the preceding paragraph it is obvious that $R_1 = 88$ per cent.

In the third volume estimation (no. 4 above) R_2 (no. 4 above) = 94 per cent. R_1 (estimated from the sample prepared in no. 3 above) is 170 per cent.

Since the hematocrit tubes receiving the blood contained no oxalate solution, C_1 and C_2 in all cases equal unity. The final results must be multiplied by $\frac{2}{3}$ because of the stronger standard used.

The results of the three volume estimations are given in the table below.

Experiment 253. The repeated blood volume method carried out in vitro on oxalated blood.

1. Three hundred cubic centimeters of blood were drawn from a normal dog into a flask containing 35 cc. of 1.6 per cent sodium oxalate. This blood was thoroughly mixed. A graduated 15 cc. hematocrit tube was filled.

2. Two hundred cubic centimeters of no. 1 were poured into a dry 250 cc. volumetric flask. Then 0.455 cc. of 1 per cent brilliant vital red was added and the flask made up to mark with no. 1. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time 14 cc. were quickly pipetted into a dry 15 cc. hematocrit tube.

3. One hundred and fifty cubic centimeters of no. 2 were then poured into a volumetric flask calibrated to hold 200 cc. Then 0.356 cc. of 1 per cent brilliant vital red was added and the flask made up to mark with no. 2. The contents of the flask were thoroughly mixed by rotation and inversion for five minutes. At the end of this time a dry graduated 15 cc. hematocrit tube was filled with the mixture. All of the hematocrit tubes were corked and centrifugalized for 30 minutes at 2500 revolutions a minute. The quantity of packed cells and of supernatant fluid in each tube was noted.

TABLE 1

Experiment 268. The repeated blood volume method carried out in vitro on oxalated blood

VOLUME DETERMINATION	VOLUME OF OXALATED PLASMA		HEMATOCRIT (CELLS)	VOLUME OF OXALATED BLOOD			TOTAL VOLUME OF PACKED CELLS	
	Actual (by hematocrit)	Estimated by dye method		Actual	Estimated by dye method	Error	Actual (by hematocrit)	Estimated by dye method
	cc.	cc.	per cent	cc.	cc.	per cent	cc.	cc.
First.....	465	454	53.5	1000	976	2.4	535	522
Second.....	271	246	53.2	510	526	3.1	266	280
Third.....	261	234	53.2	490	500	2.0	261	266

The standard solutions and dilutions employed in obtaining the colorimetric readings given below were made in the usual manner.

In the first volume estimation (in no. 2 above) $R_2 = 100$. R_1 of course in this case is zero.

In the second volume estimation (no. 3 above) $R_2 = 100$. From the preceding paragraph it is obvious that $R_1 = 100$ per cent.

Since the hematocrit tubes receiving the blood contained no oxalate solution, C_1 and C_2 in all cases equal unity.

The results of the two volume estimations are given in table 2 below.

TABLE 2

Experiment 253. The repeated blood volume method carried out in vitro on oxalated blood

VOLUME DETERMINATION	VOLUME OF OXALATED PLASMA		HEMATOCRIT (CELLS)	VOLUME OF OXALATED BLOOD			TOTAL VOLUME OF PACKED CELLS	
	Actual (by hematocrit)	Estimated by dye method		Actual	Estimated by dye method	Error	Actual (by hematocrit)	Estimated by dye method
	cc.	cc.	per cent	cc.	cc.	per cent	cc.	cc.
First.....	115	121	53.9	250	262	5.0	135	141
Second.....	92	95	53.9	200	206	3.0	108	111

Experiment 218. Repeated determination of blood volume on the same animal. Dog 19-117. Short haired, adult male bull dog. Good condition. Weight 47.5 pounds. Blood volume determinations were carried out according to the procedure already described and at the intervals indicated below:

First blood volume determination made at 10.43 a.m.

Second blood volume determination made at 11.08 a.m.

Third blood volume determination made at 11.27 a.m.

Due to the taking of blood samples for other purposes a larger amount of blood was withdrawn than is customary—a total of 108 cc.

The results of the determinations are given in table 3 below.

TABLE 3

Experiment 218. Repeated determination of blood volume on the same animal

VOLUME DETERMINATION	TIME	PLASMA VOLUME	HEMATOCRIT (CELLS)	TOTAL BLOOD VOLUME	TOTAL VOLUME OF CELLS
		cc.	per cent	cc.	cc.
First.....	10.43	1048	47.5	1994	946
Second.....	11.08	1074	45.6	1972	898
Third.....	11.27	1088	44.1	1946	858
Average.....		1070	45.7	1971	901

Experiment 227. Repeated determination of blood volume on the same animal. Dog 17-160. Short haired, brown adult male mongrel. Good condition. Weight 45 pounds. Blood volume determinations were carried out according to the procedure already described and at the intervals indicated below:

First blood volume determination made at 9.58 a.m.

Second blood volume determination made at 10.15 a.m.

The total amount removed was 45 cc.

The results of the determinations are given in table 4 below.

TABLE 4

Experiment 227. Repeated determination of blood volume on the same animal

VOLUME DETERMINATION	TIME	PLASMA VOLUME	HEMATOCRIT (CELLS)	TOTAL BLOOD VOLUME	TOTAL VOLUME OF CELLS
		cc.	per cent	cc.	cc.
First.....	9.58	1478	56.7	3413	1935
Second.....	10.15	1478	57.7	3494	2016
Average.....		1478	57.2	3454	1976

Experiment 246. Repeated determination of blood volume on the same animal. Dog 19-111. Long haired, adult male shepherd. Good condition. Weight 35 pounds. Blood volume determinations were carried out according to the procedure already described and at the intervals indicated below:

First blood volume determination made at 10.30 a.m.

Second blood volume determination made at 10.45 a.m.

Third blood volume determination made at 1.55 p.m.

Total amount of blood removed was 69 cc.

The results of the determinations are given in table 5 below.

In conclusion I wish to express appreciation to Dr. G. H. Whipple and to Mr. A. E. Belt for advice given throughout the course of this work and for valuable assistance given in the matter of arrangement and presentation of the subject matter contained in this paper.

TABLE 5

Experiment 246. Repeated determination of blood volume on the same animal

VOLUME DETERMINATION	TIME	PLASMA VOLUME	HEMATOCRIT (CELLS)	TOTAL BLOOD VOLUME	TOTAL VOLUME OF CELLS
		<i>cc.</i>	<i>per cent</i>	<i>cc.</i>	<i>cc.</i>
First.....	10.30	909	48.5	1765	856
Second.....	10.45	896	47.1	1694	798
Third.....	1.55	994	45.4	1821	827
Average.....		933	47.0	1760	827

SUMMARY

A method is outlined in which the dye blood volume method is adapted to repeated determinations on the same animal at short intervals. The soundness of the method is demonstrated by controls done *in vitro* as well as repeated determinations performed in rapid succession on the same animal.

The experimental error does not exceed 5 per cent. Fluctuations in blood volume greater than this are sometimes seen over long periods of time. These fluctuations arise from physiological factors, the exact nature of which is not yet understood.

BIBLIOGRAPHY

- (1) KEITH, ROWNTREE AND GERAGHTY: Arch. Int. Med., 1915, xvi, 547.
- (2) ROBERTSON AND BOCK: Journ. Exper. Med., 1919, xxix, 139, 155.
- (3) LAMSON: Journ. Pharm. Exper. Therap., 1915, vii, 169.
- (4) HOOPER, SMITH, BELT AND WHIPPLE: This Journal, 1920, li, page 205.

BLOOD VOLUME STUDIES

III. BEHAVIOR OF LARGE SERIES OF DYES INTRODUCED INTO THE CIRCULATING BLOOD

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Blood volume determinations by means of dye substances injected intravenously are now familiar procedures. A dye suitable for this work must be non-toxic and not permanently stored by the body cells. More important, the dye must remain in the circulating plasma at least for several minutes after its injection before its removal from the plasma is much in evidence. "Vital red" dyes have been used for blood volume determinations in human beings and animals with no untoward effects. Some of the errors which may creep into these determinations have been pointed out in other papers of this series (papers I and II). The method used in these experiments is described in detail below.

In view of the increasing interest and importance of blood volume studies it seemed desirable to study with care many dye substances which might be of value in future work—possibly of greater value than the "vital red" series. This study has been extended to include a number of dyes which are excreted in considerable measure by the kidneys or liver. In many instances the behavior of a certain dye was well known and is included in our tables mainly for comparison and control of other factors. Other dyes were tested because of a suggestive chemical constitution which would seem to indicate a possible physiological relationship to other familiar dyes. Still other dyes of this series were employed because they had been studied as to other aspects of their behavior in living animals by workers in the Department of Anatomy. It need not be repeated that at present *the behavior of a given dye in the blood stream cannot be predicted from any analysis of its chemical formula.*

We have tested by the method described below a series of over sixty dye substances. The dyes are predominantly of the benzidine series, are non-toxic and not chemically changed in the blood plasma. For the sake of convenience in tabulation these dyes have been grouped into three main divisions but it should be kept in mind that there is no sharp line of demarkation. These groups fade into each other. Group I contains dyes resembling the "vital red" series which persist in the blood plasma. Group II contains dyes which leave the blood stream rapidly and appear in the urine, for example, phenolsulphonophthalein. Group II contains dyes which leave the blood stream with considerable rapidity but do not appear to any degree in the urine. What becomes of such substances we are unable to say but it is evident that these dyes are changed by contact with the living cells so that they are no longer recognizable.

From the standpoint of blood volume the dyes of group I are of especial interest, but some significance attaches to the physiological behavior of the dyes in groups II and III. An analysis of the dyes in group I (table 5) will show a blue azo dye (T-1824) which we believe to be slightly superior to the best of the vital red series. This dye gives a blue color to the plasma which is very easy to read and can be colorimetrically determined with great accuracy. It leaves the blood plasma very slowly and hemolysis which may be present as a confusing factor in certain experiments is more easily recognized than is the case with the red dyes. Six other dye substances (table 5) may be classified with the "vital red" group for routine blood volume work and if available may be substituted for vital red.

METHOD

Unless otherwise noted a 1 per cent solution of the dye made up in distilled water and filtered is used for injection. Calculated on the basis of 2 mgm. per kilogram of body weight, the proper amount of dye is slowly injected into the jugular vein from a glass syringe. This syringe is then rinsed by withdrawing a little blood which is reinjected and followed by a little saline. Previous to the dye injection, a 10 cc. sample of blood is withdrawn in a clean 10 cc. syringe (rinsed in 0.9 per cent salt solution) and emptied into a 15 cc. graduated hematocrit tube containing 2 cc. of 1.6 per cent sodium oxalate solution. The tube is inverted twice to insure thorough mixing. Following this two 10 cc. samples of blood are withdrawn and emptied into two vaselined test

tubes. This provides serum for the standard dye mixture. To determine the rapidity of disappearance of the dye from the blood stream, samples are taken in vaselined test tubes after the injection of the dye at the following intervals and in the amounts indicated: after 2, 6, 10, 25 minutes, one 10 cc. sample; after 4, 15 and 40 minutes, two 10 cc. samples. After stoppering, the tubes are centrifuged at 2500 revolutions per minute for 30 minutes. The hematocrit readings (total volume minus 2 cc. oxalate, total cell volume and volume of red cells) are recorded. The normal serum for the standard is pipetted off into a clean dry test tube. The dyed serum samples are also pipetted off into a series of clean, dry tubes.

Standard: One-half cubic centimeter of the 1 per cent dye solution (5 mgm. dye) is made up to 100 cc. with distilled water in a volumetric flask.

Five cubic centimeters of the above dye standard are diluted with 5 cc. normal serum and 5 cc. 0.9 per cent salt solution (dilution 1 in 3). This standard reads 100 per cent.

Dilution: Tubes—Dyed plasma mixed with 0.9 per cent salt solution in the proportion 2 cc. to 4 cc. respectively (dilution 1 in 3).

Colorimetric determinations: Each of the samples of the diluted dyed serum placed successively in a colorimeter is compared with the standard in the wedge of the colorimeter and the average of five readings is recorded as the color per cent of the sample.

Computation of results: From the hematocrit readings per cent plasma and red cells may be easily obtained. It will be recalled that each 40 cc. of the standard contains 2 mgm. of the dye.

$$\text{Plasma Volume in cc.} = \frac{\text{Weight in Kilograms} \times 40}{\text{Average Colorimetric Reading}} \times 100$$

$$\text{Blood Volume in cc.} = \frac{\text{Plasma Volume}}{\text{Plasma Per Cent}} \times 100$$

Determination of amount of dye in the urine: The dog is catheterized and the bladder washed out with about 20 cc. of warm water at the beginning of the experiment. Collection of urine by catheter is made one hour after injection of the dye and at hourly intervals thereafter until the amount of dye present is too small for colorimetric determination.

Total amount of urine plus washings from each collection is measured, neutralized and made up to a volume with sufficient water to give a

color slightly less intense than the standard dye solution with which a filtered sample is compared. The volume may be 100 cc. or some multiple of 100.

Standard: One-half cubic centimeter of a 1 per cent solution of the dye, made up to 100 cc. volume with distilled water or with diluted neutral urine when specimens are not the same color as the standard aqueous solution. This standard contains 10 mgm. of dye per cubic centimeter. Whenever the first hour sample of urine apparently contains no dye, or only a trace, a portion is made acid and another alkaline to make sure that the dye is not present in some colorless form or compound easily dissociated by acid or alkali.

Dogs: For the major part of the work six normal male dogs were selected ranging in weight from 45 to 63 pounds. They were used in sequence each day so that each dog was used but once a week. This arrangement gives time for the dye to leave the plasma between determinations and also prevents the development of anemia from too frequent hemorrhage, as each experiment necessitates the withdrawal of 130 cc. of blood. The dogs are always fed after the experiment. This insures an absence of lipemia which may be a disturbing factor.

Controls for the method: For each dye duplicate experiments have been made on different dogs and with many of the dyes three to six determinations were made. Small dogs were used in addition to the large ones giving results which are consistent with those obtained for the standard dogs. Duplicate tests have been made with certain dyes on the same dog with essentially identical results.

Duplicating the blood samples for the 4, 15 and 40 minute periods gives control for the technique employed including the colorimetric readings. As a rule both tubes taken at the same time interval gave identical readings, the exception being a variation of not more than 2 per cent. To obviate the personal factor in making colorimetric readings, all determinations were made by the same observer throughout this series of experiments.

Often the samples and standard have been kept in the ice-box for a second colorimetric comparison on the following day. These late readings were practically identical with those of the freshly prepared specimens. All recorded observations, however, were made on the same day of the experiment.

Experiments using large amounts of dyes: By injecting 20 mgm. per kilogram of body weight, curves showing the rate of disappearance of dyes from the plasma have been worked out for brilliant vital red

(new); T-1824; T-1835 (alkaline); trypan blue (dyes slowly removed from the blood stream); phenolsulphonephthalein; phenoltetrachlorophthalein; crystal S. scarlet and Buffalo fast crimson (dyes rapidly removed from the blood stream). (Refer to table 11.)

The measured amount of dye (20 mgm. per kilo body weight) is slowly injected and two 10 cc. samples of blood withdrawn at 4 and 40 minutes after injection and one 10 cc. sample 1, 2, 3, 4, 6, 8 and 12 hours after injection if the dye still persists in the plasma. After 12 hours, samples are taken on successive days at approximately the same hour until the amount of dye in the plasma is too small for satisfactory colorimetric determination. Dilution of the plasma is necessary for the first specimens which are all read against the usual standard prepared as described above.

In the following list of dye substances three main groups are made as follows:

Group I. Dyes behaving in the blood stream like brilliant vital red (blood volume group).

Group II. Dyes which show rapid disappearance from the blood plasma and are excreted in large measure by the kidneys. (Type of phenolsulphonephthalein).

Group III. Dyes rapidly lost from the plasma yet not excreted by the kidneys. Certain dyes of this group show a substantial trace in the urine. (Intermediate group.)

LIST OF DYE SUBSTANCES

Group I. Blood volume group

<i>Designation</i>	<i>Chemical constitution</i>
Brilliant vital red.....	Orthotolidin + 1 mol. β naphthylamine 3.6 disulphonic acid and 1 mol. β naphthylamine 6 monosulphonic acid.
Vital new red (E and S).....	A tetrazo dye belonging to a group made by combining urea derivatives with amido naphthols, naphthols and naphthylamines.
Vital new orange (E and S).....	A tetrazo dye belonging to a group made by combining urea derivatives with amido naphthols, naphthols and naphthylamines.
No. 176.....	Tetrazo dye formed by combining para para di-amido diphenyl ureas with various naphthols, amido naphthols and naphthylamines.
Trypan red.....	Benzidine monosulphonic acid combined with 2 mol. β naphthylamine 3.6 disulphonic acid.

Dianil granat B.....	Benzidine combined with 1 mol. 2 amido-8 naphthol 6 monosulphonic acid and 1 mol. β naphthylamine 3.6 disulphonic acid.
No. 86.....	Benzidine combined with 1 mol. β naphthylamine 3.6 disulphonic acid and 1 mol. β naphthylamine 6 monosulphonic acid.
Columbia blue R.....	Benzidine combined with 1 mol. α naphthol 3.8 disulphonic acid and 1 mol. 1.8 amido naphthol 4 disulphonic acid.
No. 276.....	Benzidine combined with 1 mol. H acid and 1 mol. 2.8 ethyl amido naphthol 6 monosulphonic acid.
Direkt himmelbau gruenlich....	Dianisidine combined with 2 mol. 1.8 amido naphthol 2.5 disulphonic acid.
Chicago blue 4B.....	Dianisidine combined with 1 mol. 1.8 amido naphthol 2.4 disulphonic acid and 1 mol. 1.8 amido naphthol 4 monosulphonic acid.
Chicago blue 6B.....	Dianisidine combined with 2 mol. 1.8 amido naphthol 2.4 disulphonic acid.
No. 2826 B.....	Dianisidine combined with 1.8 amido naphthol 2.4 disulphonic acid.
Hoechst No. 9.....	Ortho tolidin combined with 2 mol. β naphthylamine 6 monosulphonic acid.
No. 204.....	Ortho tolidin combined with 1 mol. β naphthylamine and 1 mol. β naphthylamine 6 monosulphonic acid.
T-148.....	Ortho tolidin + 2 mol. α naphthylamine 4.8 disulphonic acid.
O-Tolidin + 1 NH ₂ (4.8).....	Ortho tolidin + 2 mol. α naphthylamine 4.8 disulphonic acid.
Brilliant purpurine R.....	Ortho tolidin combined with 1 mol. α naphthylamine 4 monosulphonic acid and 1 mol. β naphthylamine 3.6 disulphonic acid.
T + 2 mol. 1 naph. 3.6 disulf....	Ortho tolidin + 2 mol. α naphthylamine 3.6 disulphonic acid.
T + 2 mol. β naph. 5.7 disulf....	Ortho tolidin + 2 mol. β naphthylamine 5.7 disulphonic acid.
T-1824.....	Ortho tolidin combined with 2 mol. 1.8 amido 2.4 disulphonic acid.
No. 2826 A.....	Ortho tolidin combined with 2 mol. 1.8 amido 2.4 disulphonic acid.
2 mol. 1824 - 1 mol. 0-tolidin....	Ortho tolidin combined with 2 mol. 1.8 amido naphthol 2.4 disulphonic acid.
Dianil blue 2 R.....	Ortho tolidin combined with 1 mol. chromatrope acid and 1 mol. α naphthol 4 monosulphonic acid.
T + 1835 (alkaline).....	Ortho tolidin combined with 2 mol. 1.8 amido naphthol 3.5 disulphonic acid in alkaline solution.

T + 2 mol. 1846.....	Ortho tolidin combined with 2 mol. 1.8 amido naphthol 4.6 disulphonic acid.
Columbia blue G.....	Ortho tolidin combined with 1 mol. α naphthol 3.6 disulphonic acid and 1 mol. 1.8 amido naphthol 4 monosulphonic acid.
Wasserblau.....	Sulphonic acid of triphenyl para rosaniline.
Indazarun B. B.....	Dianisidine combined with 1 mol. 1.7 dioxy-2 naphtholic acid-4 sulphonic acid and 1 mol. β naphthol 3.6 disulphonic acid.
No. 173.....	Tetrazo dye formed by combining para para diamido diphenyl ureas with various naphthols, amido naphthols and naphthylamines.

Group II. Renal excretion group

Phenolsulphonephthalein

Buffalo fast crimson..	Mono azo dye formed by linkage of acetyl β phenylendiamine combined with α naphthol 3.6 disulphonic acid.
Crystal s. scarlet.....	Mono azo dye formed by combining α naphthylamine with β naphthol 6.8 disulphonic acid.
Tolan red.....	Mono azo dye formed by combining aniline with 1.8 amido naphthol 4.6 disulphonic acid.
No. 226.....	Benzidine disulphonic acid with 2 mol. 1.8 amido naphthol 4.6 disulphonic acid.
No. 227.....	Benzidine disulphonic acid with 2 mol. 2.8 amido naphthol 6 monosulphonic acid.
No. 228.....	Benzidine disulphonic acid with 2 mol. 2.5 naphthol 7 monosulphonic acid.
T-1835.....	Ortho tolidin combined with 2 mol. 1.8 amido 3.5 disulphonic acid (probably in acid solution).
No. 225.....	Benzidine meta disulphonic acid + 2 mol. H acid.
No. 105.....	Benzidine combined with 2 mol. chromatrope acid.
L. T. 297.....	Ortho tolidin disulphonic acid combined with 2 mol. amido naphthol 3.6 disulphonic acid.
T. disulfosaure + H acid.....	Ortho tolidin disulphonic acid combined with 2 mol. amido naphthol 3.6 disulphonic acid.
Alizarin green S.....	A mixture of tri and tetra oxy anthraquinone quinolines and their sulphonic acid derivatives.
No. 155.....	Para para diamido stilbene combined with 2 mol. H acid.
Indigo disulfosaure.....	Indigo disulphonic acid.

Group III. Intermediate group

New Bordeaux L.....	Benzidine combined with 2 mol. β naphthol 8 monosulphonic acid.
Baumwoll rubin.....	Benzidine combined with 1 mol. α naphthylamine 4 monosulphonic acid and 1 mol. β naphthol 8 monosulphonic acid.
Naphthamine black C. E.....	Benzidine combined with 1 mol. of 2.8 amido naphthol 6 monosulphonic acid and 1 mol. H acid.
Naphthamine black R. E.....	Benzidine combined with 1 mol. 2 amido 8 naphthol 6 monosulphonic acid + 1 mol. of the K acid.
Trisulfon violet.....	Benzidine combined with 1 mol. β naphthol and 1 mol. α naphthol 3.6.8. trisulphonic acid.
No. 114.....	Benzidine combined with 1 mol. H acid and 1 mol. α naphthol 4 monosulphonic acid.
No. 316.....	Benzidine combined with 1 mol. H acid and 1 mol. 2.8 ethyl amido naphthol 6 monosulphonic acid.
No. 221.....	Dianisidine combined with 1 mol. Neville-Winther acid and 1 mol. 1.7 dioxy 2 naphthoe 4 sulphonie acid.
No 295.....	Dianisidine combined with 1 mol. α naphthol 4 monosulphonic acid and 1 mol. 1.7 dioxy 2 naphthoe 4 disulphonic acid.
No. 481 Mulheim blue II..	Ortho tolidin combined with 2 mol. chromotrope acid.
Lichtgrün S. F.....	Triphenyl methane dye. Chlor methylate of hexa methyl para rosaniline chloride.
Trypan blue.....	Benzidine dye: Ortho tolidin combined with 2 mol. 1.8 amido naphthol 3.6 disulphonic acid.
Congo blue (X pure)	Ortho tolidin combined with 1 mol. α naphthol 4 monosulphonic acid and 1 mol. H acid.
No. 222.....	Dianisidine combined with 1 mol. H acid and 1 mol. 1.7 dioxy 2 naphthoeic acid 4 sulphonic acid.
No 181.....	Tetrazo dye formed by combining para para diamido diphenyl ureas with various naphthols, amido naphthols and naphthylamines.
No. 230.....	Benzidine disulphonic acid combined with 2 mol. β naphthylamine 7 monosulphonic acid.
No. 246.....	Tetrazo dye formed by combining para para diamido diphenyl ureas with various naphthols, amido naphthols and naphthylamines.

EXPERIMENTAL OBSERVATIONS

It will be seen from table 1 that the different vital red dyes show considerable differences in the color intensities of the standard 1 per cent solution. Whether this may be due in part to inert salts present in the dye powder is not determined. It will be seen that the dyes may differ slightly in their physiological reaction in the plasma (table 3). For example, brilliant vital red II leaves the circulation with slightly greater speed than some of the other vital red dyes.

TABLE 1
Comparison of vital red dyes

DESIGNATION	CHEMICAL CONSTITUTION	COLOR STRENGTH
		<i>per cent</i>
Brilliant vital red (new)	Ortho tolidin combined with one molecule β naphthylamine 3.6 disulphonic acid and one molecule β naphthylamine 6 monosulphonic acid	100
Brilliant vital red (old)	Ortho tolidin combined with one molecule β naphthylamine 3.6 disulphonic acid and one molecule β naphthylamine 6 monosulphonic acid	75
Brilliant vital red II	Ortho tolidin combined with one molecule β naphthylamine 3.6 disulphonic acid and one molecule β naphthylamine 6 monosulphonic acid	50
Original Rowntree dye	Diamino dixylyl methane combined with two molecules β naphthol 3.6 disulphonic acid	60
Rowntree II no. 273	Dichlor benzidine combined with 2 molecules β naphthylamine 3.6 disulphonic acid	100

Standardization of the six large dogs by means of these vital red dyes is detailed in table 2. It will be noted that these standard observations were made at intervals during the period in which these dogs were used to standardize the other dyes as indicated in other tables to follow. Table 2 may be used as a reference table to show the average blood volume figures as well as maximal and minimal readings for blood volume in individual dogs as determined by the vital red dye group. These same standardized dogs are used throughout the entire series of observations tabulated below. It is interesting to note the average figure for blood per kilo equals 93 and average cell hematocrit equals 50.4 per cent. It

may be stated again that these dogs were all strong, well-nourished, rather inactive adult males.

The speed with which these vital red dyes are removed from the circulating plasma can be estimated from the color readings given in table 3. During a period of 40 minutes on the average the color readings fall 13 per cent—that is, from an average reading of 90 color per cent to an average of 77 color per cent. There is good evidence that

TABLE 2
Blood volume determinations with vital red dyes

DATE	DYE	DOG	WEIGHT	RED CELLS	PLASMA	COLOR (4 MIN.)	PLASMA VOL- UME	BLOOD VOL- UME	BLOOD PER KILOGRAM
			kgm.	per cent	per cent	per cent	cc.	cc.	
11/18	Brilliant vital red (new).....	16-178	27.04	43.2	55.1	89	1215	2206	82
11/26	Brilliant vital red (old).....	16-178	27.10	46.4	53.1	99	1095	2063	76
2/27	Brilliant vital red II.....	16-178	28.45	46.6	52.4	94	1210	2309	81
12/15	Original Rowntree dye.....	16-178	26.95	44.0	54.5	99	1088	1996	74
12/30	Brilliant vital red (new).....	18-60	21.90	48.6	50.4	83	1055	2093	91
11/22	Brilliant vital red (old).....	18-60	22.30	51.2	47.7	95	939	1968	88
11/29	Original Rowntree dye.....	18-60	21.80	52.7	46.3	82	1063	2295	105
2/25	Original Rowntree dye.....	18-60	21.90	47.5	51.5	76	1152	2236	102
11/21	Brilliant vital red (new).....	19-63	21.60	50.0	48.8	80	1080	2213	102
11/30	Brilliant vital red (old).....	19-63	21.90	53.6	45.4	87	1006	2215	101
12/28	Original Rowntree dye.....	19-63	23.00	50.2	48.6	80	1152	2370	102
11/23	Brilliant vital red (new).....	19-70	21.30	52.8	46.1	94	906	1965	92
4/29	Brilliant vital red II.....	19-70	26.80	45.8	53.7	99	1083	2017	75
5/6	Brilliant vital red (new).....	17-154	26.90	54.4	45.1	96	1123	2490	92
4/30	Brilliant vital red II.....	17-154	27.00	53.8	45.2	95	1137	2515	93
3/4	Brilliant vital red (new).....	19-71	24.10	53.4	46.1	96	1004	2193	91
1/22	Brilliant vital red II.....	19-71	23.50	54.8	45.2	86	1093	2418	103
2/11	Rowntree II no. 273.....	19-71	23.50	51.4	47.6	81	1160	2437	104
3/31	Rowntree II no. 273.....	19-71	25.50	51.7	47.2	88	1160	2457	96
Average.....			24.4	50.4	48.6	89			93

the dye is thoroughly mixed with the circulating blood at the end of 2 minutes as these readings are so constant when compared with the 4-minute samples which average 1 color per cent less. All the blood volume determinations are figured from the 4-minute reading which is arbitrarily taken as the optimum figure.

The data on color concentration in blood plasma at varying time intervals are presented in table 3 but the same data are given in different

TABLE 3
Color determinations vital red dyes readings in per cent

DYE (DESIGNATION).....	BRILLIANT VITAL RED (NEW)						BRILLIANT VITAL RED (OLD)		BRILLIANT VITAL RED II				ORIGINAL ROWNTREE DYE			ROWNTREE DYE II	
	16-178	13-60	19-63	19-70	17-154	19-71	18-40	19-63	16-178	19-70	17-174	19-71	18-60	18-60	19-63	19-71	19-71
Dog number.....	59.5	48	47.5	47.0	59	53	49.13	48.25	62.5	59	59.5	51.5	48.0	48.13	50.75	51.75	56.0
Weight, pounds.....																	
Time after injection readings in per cent	90	85	88	95	97	97	99	90	94	100	95	88	85	77	80	81	90
	89	83	86	94	96	96	95	87	94	99	95	86	82	76	80	81	88
	88	83	84	94	95	96	88	86	93	97	93	85	82	75	79	81	87
	84	83	80	94	93	95	86	83	91	95	88	83	81	73	77	80	85
	83	82	77	90	90	93	85	80	88	92	85	81	80	71	75	78	83
	81	80	73	86	88	91	83	78	81	87	81	74	77	70	71	76	80
40 min.	79	79	70	79	87	89	79	76	76	83	77	70	73	68	68	75	77
Total loss in color per cent—40 minutes.....	11	6	18	16	10	8	20	14	18	17	18	18	12	9	12	6	13

form in table 4 to show the loss in color per cent reading during the time intervals as tabulated. There is practically always a slight fall in color readings in the interval between the 2- and 4-minute samples (table 4—first column, 2 to 4)—average 1.4 color per cent. The same fall is noted in the interval between the 4- and 6-minute samples—average 1.2 color per cent. The loss of dye from the plasma is less

TABLE 4

Decrease in color concentration of plasma; minutes after injection

DYE (DESIGNATION)	DOG	2 TO 4 MIN- UTES	4 TO 6 MIN- UTES	6 TO 10 MIN- UTES	10 TO 15 MIN- UTES	15 TO 25 MIN- UTES	25 TO 40 MIN- UTES	TOTAL 40 MIN- UTES COLOR PER CENT
Brilliant vital red (new) . . .	18-60	2	0	0	1	2	1	6
	19-63	2	2	4	3	4	3	18
	16-178	1	1	4	1	2	2	11
	19-70	1	0	0	4	4	7	16
	17-154	1	1	2	3	2	1	10
	19-71	1	0	1	2	2	2	8
Brilliant vital red (old)	18-60	4	7	2	1	2	4	20
	19-63	3	1	3	3	2	2	14
Brilliant vital red II	16-178	0	1	2	3	7	5	18
	19-70	1	2	2	3	5	4	17
	17-154	0	2	5	3	4	4	18
	19-71	2	1	2	2	7	4	18
Original Rowntree	18-60	3	0	1	1	3	4	12
	18-60	1	1	2	2	1	2	9
	19-63	0	1	2	2	4	3	12
Rowntree II no. 273	19-71	0	0	1	2	2	1	6
	19-71	2	1	2	2	3	3	13
Average		1.4	1.2	2.1	2.2	3.3	3.1	13.3

rapid as we approach the 40-minute period—for example, a loss of 3.1 color per cent during 15 minutes preceding the 40-minute reading. During the first 10 minutes after the dye injection there is an average loss in color per cent reading of 1 per cent per 2-minute intervals. It will be noted that brilliant vital red (old) and (II) leave the blood stream a little more rapidly on the average than do the other dyes in this group. A part of this difference may be apparent and not real.

TABLE 5
Color determinations for dyes of group I A: readings in per cent

DYE	T-1824			TWO MOLE- CULES 1824		No 2826A	T-1835 (ALKALINE)		CHICAGO BLUE OB		DIANIL BLUE 2R		No. 2836B		HOECHST 9		No. 173	
	18-60	19-63	17-154	19-63	19-71		17-154	18-60	18-00	19-71	19-70	19-71	19-70	17-154	16-178	19-70	19-71	18-60
Dog number.....	48	49-25	61-0	49-25	55-0	60-5	59-63	47-38	47-0	49-5	50-35	51-0	47-5	58-75	63-13	45-63	56-5	51-5
Weight in pounds.....	78	83	98	90	97	104	90	76	76	97	92	84	97	110	87	74	98	85
Time after injection reading in per cent.....	2 min.	78	83	90	96	102	89	75	75	97	91	83	96	110	87	73	97	84
	4 min.	78	82	96	89	100	89	74	74	96	90	82	95	108	87	73	96	82
	6 min.	77	80	95	87	99	87	74	73	74	95	88	81	88	106	85	71	94
	10 min.	77	79	95	83	91	86	72	71	72	95	86	80	88	106	82	67	90
	15 min.	77	75	93	78	88	84	70	68	71	94	84	75	84	106	75	58	80
Total loss in color per cent in 40 minutes.....	25 min.	70	74	91	74	85	83	69	64	68	93	81	70	83	104	71	54	72
	40 min.	8	9	7	16	12	7	7	8	4	11	14	14	6	16	20	15	13

These two dyes are paler than the others and the color readings therefore a little less accurate. The differences, however, are too small to have much significance.

In group I-A are included seven dyes which compare favorably with the vital red series and may be substituted if occasion arises. There are five blue dyes in this group and the blue color in itself has certain advantages over the red dye colors. Many workers can estimate blue colors more accurately, more rapidly and with less fatigue than is the case with red colors. Slight hemolysis can be recognized more easily when the blue dyes are used. Even a trace of hemolysis will rarely confuse one who is thoroughly familiar with the blood volume work no matter what dye is being used.

One blue dye is particularly well adapted to the routine blood volume work and is preferred by us when compared with the vital red group. The difference, however, is not great but for many workers the blue dye has advantages. This azo dye is tested in table 5 in seven different experiments. Three different samples of the same dye are used (T-1824 and 2-mol. 1824, 1 mol O-tolidin and No. 2826 A) and tabulated in the first three groups of table 5. It will be noted that this blue dye is slow to leave the circulating blood plasma, if anything slightly more so than the vital red series.

The second blue dye (T-1835 alkaline) is also slow to leave the blood stream during the observed period of 40 minutes but the three color readings are somewhat lower than the usual average. We are not prepared to explain this point but many more observations must be made before any such unusual reaction can be accepted.

AVERAGE COLOR CONCENTRATION	VITAL RED	T-1824	T-1835 (ALKALINE)
Two-minute samples.....	90	94	81
Four-minute samples	88	93	80
Forty minute samples.....	78	84	72

The above tabulation shows that the vital red figures compare closely with the blue azo dye T-1824. The readings of T-1835 (alkaline) are low but these figures represent the average of too few observations while the vital red figures represent a great many experiments.

It will be seen that the curve of dye removal from the blood stream is the same for this group of dyes as for the vital red group. If anything these dyes (group I-A) are even more slowly removed from the blood stream, particularly the blue dyes.

The two red dyes (Hoechst 9 and No. 173) of group 1-A behave very much like the vital red group. Too few observations have been made to determine this point beyond question.

TABLE 6
Decrease in color concentration of plasma for dyes of group I-A.
Minutes after injection

DYE	DOG	2 TO 4 MIN-UTES	4 TO 6 MIN-UTES	6 TO 10 MIN-UTES	10 TO 15 MIN-UTES	15 TO 25 MIN-UTES	25 TO 40 MIN-UTES	TOTAL 40 MIN-UTES COLOR PER CENT
T-1824.....	18-60	0	0	1	0		7	8
	19-63	0	1	2	1	4	1	9
	17-154	0	2	1	0	2	2	7
2 mol. 1824, 1 mol. 0-tolidin	19-63	0	1	2	4	5	4	16
	19-71	1	1	2	2	3	3	12
No. 2826 A.....	17-154	2	2	1	2	2	2	9
	16-178	2	2	2	1	0	1	8
T-1835 (alkaline).....	17-154	1	0	2	1	2	1	7
	18-60	1	1	0	2	2	1	7
	19-63	1	1	1	2	3	4	12
Chicago blue 6B.....	18-60	1	0	1	2	1	3	8
	19-71	0	1	1	0	1	1	4
Dianil blue 2R.....	19-71	1	1	1	1	5	5	14
	19-70	1	1	2	2	2	3	11
No. 2826 B.....	19-70	1	1	7	0	4	1	14
	17-154	0	2	2	0	0	2	6
Hoechst 9.....	16-178	0	0	2	3	7	4	16
	19-70	1	0	2	4	9	4	20
No. 173.....	19-71	1	1	2	4	2	5	15
	18-60	1	2	1	1	3	5	13

The corresponding blood volumes on the standardized dogs are figured in table 7 and are found to be in harmony with observations made with vital red dyes in table 2. The first experiment in table 7 gives a figure of 70 cc. per kilo for the blood volume which is open to

criticism. This figure is very low as compared with many others on this same dog and is much below the average. It is more than probable that some technical error crept into this experiment—possibly some dye contaminating a container, syringe or hypodermic needle.

Group I-B contains many dyes which are fairly satisfactory in many respects when used for blood volume determinations. These dyes do not leave the blood stream rapidly and in only one or two instances

TABLE 7
Blood volume determinations group I-A

DATE	DYE	DOG	WEIGHT	RED CELLS	PLASMA	COLOR PER CENT 4 MINUTES	PLASMA VOL-UME	BLOOD VOL-UME	BLOOD PER KILOGRAM	DECREASE IN COLOR CONCENTRATION 40 MINUTES
			<i>kgm.</i>	<i>per cent</i>	<i>per cent</i>		<i>cc.</i>	<i>cc.</i>		
1/15	No. 2826A	16-178	27.5	44.2	54.8	104	1058	1930	70	9
1/2	Hoechst No. 9	16-178	28.2	48.1	50.9	87	1298	2550	90	16
12/9	T + 1824	18-60	21.8	50.2	48.8	78	1120	2295	105	8
2/3	T + 1835 (alkaline)	18-60	21.5	49.5	49.5	75	1149	2321	107	7
1/7	Chicago 6 B	18-60	21.3	49.5	50.0	75	1138	2276	106	8
6/30	No. 173	18-60	23.4	48.4	50.1	84	1116	2227	95	13
12/7	T + 1824	19-63	22.4	52.6	45.8	83	1079	2356	104	9
1/22	2 mol. 1824 + 1 mol. 0-tolidin	19-63	22.4	54.0	45.0	90	995	2211	98	16
3/1	T + 1835 (alkaline)	19-63	23.7	51.0	48.0	75	1266	2637	111	12
2/5	Dianil blue 2 R	19-70	23.7	46.1	52.9	91	1044	1973	83	11
1/11	No. 2826 B	19-70	21.6	43.7	55.3	96	900	1627	96	14
1/4	Hoechst No. 9	19-70	20.2	45.8	53.2	73	1109	2084	102	20
3/3	T + 1824	17-154	27.7	54.5	44.5	98	1132	2544	92	7
1/10	No. 2826 A	17-154	27.5	54.6	44.4	102	1078	2428	88	9
2/1	T + 1835 (alkaline)	17-154	27.1	60.5	39.5	89	1195	3026	111	7
1/16	No. 2826 B	17-154	26.7	62.5	36.5	110	970	2657	99	6
5/2	2 mol. 1824 + 1 mol. 0-tolidin	19-71	25.0	53.8	45.7	96	1042	2280	91	12
1/14	Chicago blue 6 B	19-71	22.5	55.6	43.4	97	928	2138	95	4
2/4	Dianil blue 2 R	19-71	23.2	55.2	44.3	83	1118	2523	109	14
6/10	No. 173	19-71	25.7	57.2	41.8	97	1059	2533	99	15

are moderately toxic. As a group the colors are pale and correspondingly difficult to read. A pale color cannot be as accurately determined and work with such dyes is more time consuming. It should be stated, however, that trypan red, indazarun B. B. and no. 204 when injected in the strength of 4 mgm. per kilo do give satisfactory colors for routine work. Used in this strength these three dyes compare favorably with the vital red group.

TABLE 8
Blood volume determinations, group I-B

DATE	DYE	DOG	WEIGHT	RED CELLS	PLASMA	COLOR PER CENT 4 MINUTES	PLASMA VOL- UME	BLOOD VOL- UME	BLOOD PER KILOGRAM	DECREASE IN COLOR CONCENTRATION 2-40 MINUTES
			<i>kgm.</i>	<i>pct cent</i>	<i>pct cent</i>		<i>cc.</i>	<i>cc.</i>		
10/14	Vital new red	16-178	28.0	45.5	53.4	104	1077	2016	72	21
1/8	Chicago blue 4B	16-178	28.0	48.8	50.2	104	1077	2145	76	15
1/23	No. 204	16-178	28.5	45.5	53.5	98	1163	2173	76	22
1/30	Indazarun B.B.	16-178	28.2	50.9	49.0	97	1163	2373	84	11
1/17	Vital new red	18-60	21.2	49.5	50.0	87	977	1940	91	8
5/15	No. 176	18-60	24.8	46.3	52.7	91	1090	2068	82	10
4/26	No. 86	18-60	24.5	45.3	53.7	82	1195	2225	90	10
3/5	No. 204	18-60	22.1	49.0	50.5	79	1119	2215	100	18
3/22	T - 148	18-60	23.0	47.1	51.9	93	989	1905	83	8
12/16	T + 2 mol. β naphth. 5.7 disulph.	18-60	21.5	50.2	48.7	85	1011	2076	96	13
2/21	Trypan red	19-63	23.0	50.3	48.7	73	1263	2593	112	12
12/14	Direkt himmel blau gruenlich	19-63	23.0	50.7	47.9	79	1167	2436	105	13
1/13	Chicago blue 4 B	19-63	22.4	55.8	43.2	87	1030	2384	106	8
12/21	T + 2 mol. 1846	19-63	23.8	49.5	49.5	72	1325	2676	113	8
1/31	Indazarun B.B.	19-63	21.7	54.1	44.9	77	1114	2481	114	14
6/27	No. 176	19-70	25.7	57.0	41.9	114	901	2150	83	14
2/19	Trypan red	19-70	23.6	42.2	56.8	93	1017	1790	75	8
12/24	T + 2 mol. 1 naph. 3.6 disulf.	19-70	21.3	35.3	63.7	83	1265	1985	93	23
12/17	T + 2 mol. 1 naph. 3.6 disulf.	19-70	21.7	44.8	54.2	92	945	1743	80	9
	No. 86	17-154	27.5	53.2	45.8	95	1157	2526	92	7
6/28	No. 276	17-154	27.7	58.6	40.4	99	1122	2777	100	21
12/13	Direkt himmelblau gruenlich	17-154	27.7	55.5	43.4	97	1144	2635	94	5
12/20	2 mol. β naph. 5.7 di-sulfosaure	17-154	27.7	55.7	43.7	102	1088	2489	89	14
4/16	Wasserblau	17-154	27.0	53.2	46.2	85	1165	2502	92	8
5/16	No. 276	19-71	25.2	52.2	47.2	90	1122	2377	94	18
3/25	T - 148	19-71	25.0	52.8	46.7	92	1087	2327	93	7
12/18	T + 2 mol. 1846	19-71	23.6	51.1	48.3	79	1197	2478	104	5
4/15	Wasserblau	19-71	25.3	53.8	45.7	100	1014	2219	87	13

Wasserblau is decolorized by the blood plasma but the color returns on the addition of 2 drops of concentrated hydrochloric acid to blood serum tubes and standard. Brilliant purpurine in the strength of 4

mgm. per kilo will cause hemolysis in the circulating blood, and some clinical evidence of intoxication.

Vital new red, vital new orange, direkt himmelblau gruenlich and T-148 persist in the blood stream for 7 to 10 days or longer. This is an objection to the routine use of these four dyes and may introduce difficulties when a series of observations is to be made upon the same animal.

As regards the dyes in group I-B, too few observations have been made to settle all points of interest which concern blood volume work. Some single observations are not even included in table 8 as they show nothing unusual and the dyes gave no promise of being useful in this work. This statement applies to columbia blue R, O-tolidin+1 NH₂ (4.8), dianil granat B, brilliant purpurine R, columbia blue G, and some others listed but not specifically mentioned.

TABLE 9
Summary. Dyes of group I

	VITAL RED DYES 19 EXPERIMENTS			OTHER DYES OF GROUP I-A 20 EXPERIMENTS			DYES IN GROUP I-B 28 EXPERIMENTS		
	Aver- age	Mini- mum	Maxi- mum	Aver- age	Mini- mum	Maxi- mum	Aver- age	Mini- mum	Maxi- mum
Kilogram weight.....	24.4	21.3	28.45	24.1	21.3	28.25	24.7	21.2	28.5
Red cells per cent.....	50.4	43.2	54.8	51.8	43.7	62.5	50.1	35.3	58.6
Color per cent (4-minute sample)	89	76	99	89	73	110	90.3	72	114
Blood per kilogram body weight	93	74	105	92	70	111	92	72	114
Decrease in color per cent 2-40 minutes	13	6	20	11	6	20	12	5	23

Dyes of group II

This group includes the dyes which are eliminated more or less completely by the kidneys. Phenolsulphonephthalein is a dye familiar to everyone and we record in table 10 familiar data concerning renal excretion of this dye. The amounts injected are somewhat larger than the usual clinical dose but the per cent excretion is normal for dogs. The rapid disappearance from the blood stream is well shown. Much of the phthalein has left the blood by the end of 2 minutes and almost all of it by the end of the next 4 minutes. Phenoltetrachlor-phthalein (1) which is known to be excreted in the bile is taken out of

the blood under similar conditions with even greater speed. There is practically complete removal of this dye from the blood serum by the end of 2 minutes following its injection. Perhaps the relatively greater size of the liver and consequent larger minute circulation volume as compared with the kidneys may account for this difference.

Several of the dyes listed in table 11 (group II) are rapidly removed from the blood stream but none with the speed just recorded for phenol-sulphonephthalein. Buffalo fast crimson, crystal S. scarlet, tolan red, T-1835 and indigo disulfosauare are dyes which show minimal color readings in the plasma 6 minutes after the dye injection. Probably the dye is almost completely removed within a period of 15 minutes.

TABLE 10

*Decrease in color concentration of plasma and excretion by the kidneys—
Phenolsulphonephthalein**

DATE	DOG	WEIGHT	COLOR PER CENT 2 MINUTE SAMPLE	LOWEST COLOR PER CENT RECORDED	TIME INTERVAL OF LOWEST COLOR PER CENT	TOTAL DYE RECOV- ERED IN URINE	TIME RECOVERY OF DYE IN URINE
		<i>kilograms</i>			<i>minutes</i>	<i>per cent</i>	<i>hours</i>
5/7	16-178	24.65	26	18	4	62.8	4
5/8	18-60	24.5	38	27	4	72.7	4
5/7	19-63	22.8	28	20	4	60.5	6
12/10	19-70	21.3	41	28	4	60.0	2
12/6	17-154	27.0	50	38	4	70.3	3
5/8	19-71	25.5	35	23	4	64.9	4
Average		24.3	36	26		65.2	

* 1.3 mgm. per kilo body weight.

Buffalo fast crimson seems to be more like phenolsulphonephthalein in that over 50 per cent of the dye is excreted in the urine. It seems that this dye should be more carefully studied as to its excretion by the kidney in health and disease. The other dyes show wide variations in the amount recovered from the urine after intravenous injection. It is possible that conditions present in the kidney, bladder or urine may modify the yield to a considerable extent. A more careful study of this group of dyes should be undertaken and may yield information of value concerning the secretory activity of normal and abnormal cells as concerns these particular dyes. It will be of some interest to determine whether the same or different limitations apply to all these dyes as is true for phenolsulphonephthalein.

The other dyes in group II leave the blood stream less rapidly and in most instances a readable amount of dye remains at the end of 40

TABLE 11
Dyes of group II

DATE	DYE	DOG	WEIGHT	COLOR PER CENT 2 MIN- UTES	LOWEST COLOR PER CENT RE- CORDED	TIME INTERVAL OF LOWEST COLOR PER CENT	TOTAL DYE RE- COVERED IN URINE	URINE COLLEC- TION
			kgm.			minutes	per cent	hours
12/12	T + 1835 (B 1841)	16-178	27.0	48	33	6	21.1	4
12/14	L. T. 297	16-178	27.75	78	54	40	14.4	3
11/1	Alizarin green	16-178	26.76	61	39	45	24.8	3
3/6	No. 227	16-178	29.0	65	36	40	8.1	6
5/23	Buffalo fast crimson	18-60	25.0	61	35	15	53.0	3
11/13	Crystal s. scarlet	18-60	22.3	63	44	6	20.6	2
4/12	Indigo disulfosäure	18-60	23.8	42	20	6	53.4	3
12/23	L. T. 297	18-60	22.2	77	54	40	15.2	4
4/19	No. 225	18-60	24.5	65	36	40	17.2	4
1/2	No. 228	18-60	21.3	73	48	40	8.8	4
6/7	Crystal s. scarlet	19-63	23.6	66	30	15	5.3	1
4/4	Indigo disulfosäure	19-63	23.5	55	35	6	22.3	4
1/6	No. 105	19-63	23.2	69	44	40	4.0	2
4/25	No. 225	19-63	24.0	55	23	40	14.3	4
3/4	No. 227	19-63	21.9	65	36	40	11.7	6
12/2	T - disulfosäure + H. acid	19-70	20.3	74	47	40	6.6	4
1/29	No. 226	19-70	21.9	72	37	40	20.7	6
11/27	T - 1835	17-154	27.1	50	37	6	15.4	4
1/3	No. 105	17-154	27.2	86	60	40	18.2	2
1/25	No. 228	17-154	26.9	75	55	40	6.2	3
12/3	Buffalo fast crimson	19-71	22.9	58	28	15	51.1	3
1/7	Tolan red 4 B	19-71	23.3	59	31	10	29.2	4
12/31	Tolan red 4 B	19-71	23.3	52	30	10	29.5	4
3/21	T + 2 mol. 1.8 amido naph. 3.5 disulfosäure	19-71	25.0	53	28	6	7.5	4
12/11	T + 1835 (B. 1841)	19-71	23.3	50	35	4	13.7	4
11/25	T + 1835	19-71	24.3	44	32	6	7.2	4
11/15	Alizarin green	19-71	23.4				16.0	1
1/28	No. 226	19-71	23.3	73	41	40	19.1	5

minutes. One is not surprised therefore to note that the dye elimination in the urine is scanty and rarely exceeds 15 to 20 per cent.

Group III-A

This group contains a number of dyes which exhibit peculiar reactions in the blood stream when compared with the dyes in group I. At present we have insufficient experimental data to give the correct explanation so that discussion of various possibilities will be brief.

Two dyes (new Bordeaux L and lichtgrün S. F.) leave the blood stream with considerable rapidity yet do not appear in the urine (table

TABLE 12
Dyes of group III-A

DATE	DYE	DOG	WEIGHT	COLOR CONCEN- TRATION 2 MINUTES	LOWEST COLOR PER CENT RECORDED	TIME INTERVAL FOR LOWEST COLOR PER CENT
			<i>kgm.</i>	<i>per cent</i>		<i>minutes</i>
3/26	New Bordeaux L	16-178	27.0	62	35	10
3/20	Baumwoll rubin	16-178	27.0	86	48	40
3/13	Naphthamine black R. E.	16-178	28.3	77	41	40
3/19	New Bordeaux L	18-60	22.3	70	37	10
2/17	Naphthamine black R. E.	18-60	21.95	55	44	40
4/5	No. 413 trisulfon violet	18-60	24.5	50	40	40
4/11	Lichtgrün S. F.	19-63	24.0	75	43	10
4/18	No. 114	19-63	24.0	94	66	40
6/24	No. 316	19-63	24.1	68	34	40
6/30	No. 295	19-63	24.5	75	52	40
4/3	Mulheim blue	19-70	26.5	99	69	40
4/10	No. 413 trisulfon violet	19-70	26.7	55	33	40
4/17	No. 114	19-70	27.0	100	73	40
3/24	Baumwoll rubin	17-154	27.0	87	55	40
4/9	Mulheim blue	17-154	27.6	110	85	40
2/15	No. 221	17-154	27.5	99	70	40
3/10	No. 316	17-154	27.0	72	33	40
6/9	No. 295	17-154	27.5	95	71	40
4/8	Lichtgrün S. F.	19-71	25.25	79	39	10
3/	No. 221	19-71	24.0	94	55	25
2/17	Naphthamine black R. E.	19-71	24.0	82	54	40

12). We have not been able to test for elimination in the bile so this cannot be excluded. Lichtgrün S. F. was decolorized in the blood plasma. Two drops of acetic acid brought back the color but hydrochloric acid did not develop the color (compare wasserblau). New Bordeaux L. was tested *in vitro* against normal serum which did not influence the dye even after three days' incubation at 38°C. It is possible, of course, that the endothelial cells of the living body are concerned in this reaction.

Other dyes in table 12 show occasionally a figure for the 40-minute period indicating a slow removal but a low initial reading. This, however, is not constant and the low initial reading is perhaps an accident due to unknown factors. There is a possibility of dye removal by the process of coagulation—that is, the dye may be carried out of solution by the clot. Unfortunately this point has not been controlled in this group as has been done in the vital red group. When these experiments were begun this possibility was not considered and oxalate plasma was not used in place of coagulated serum. In view of the irregular figures noted in table 12 for the same dye we must consider this possibility until it is disproved.

TABLE 13

*Decrease in color concentration of plasma. Group III-B.
Minutes after injection*

DATE	DYE	DOG	2 TO 4 MIN-UTES	4 TO 6 MIN-UTES	6 TO 10 MIN-UTES	10 TO 15 MIN-UTES	15 TO 25 MIN-UTES	25 TO 40 MIN-UTES	TOTAL 40 MIN-UTES COLOR PER CENT
2/6	Congo blue B	16-178	2	0	2	3	13	5	25
2/20	No. 230	16-178	1	3	6	3	9	6	28
3/11	No. 230	18-60	4	3	4	3	4	6	24
6/25	No. 181	18-60	2	4	3	5	5	13	32
5/3	Trypan blue	19-63	3	3	6	3	3	6	24
2/7	Congo blue B	19-63	1	3	2	2	6	5	19
5/14	No. 246	19-63	4	3	7	5	5	5	29
5/12	No. 181	19-70	0	8	3	4	20	6	41
2/26	Trypan blue	19-71	0	1	2	3	4	12	22
6/24	No. 246	19-71	1	3	5	8	8	5	30

The dyes given in table 13 all give a rather low initial reading as well as a pretty rapid removal during the 40-minute period. It is possible at least that the endothelial cells are concerned in a part of this reaction. It should be noted that the urine usually shows a definite trace of dye but not enough for a colorimetric reading. Three dyes not tabulated have been tested (no. 222, Hoechst no. 229 and no. 258). All these dyes give colors in the blood serum which are too pale to be accurately estimated.

When large amounts of dye are injected into the blood stream (10 to 20 times the routine amount) the reaction is slightly different but the dyes of group I give constant figures (table 14). The vital red dyes are removed from the blood with somewhat more speed than the blue

dyes (T-1824 and T-1835, alkaline). Traces of the dye remain in the serum for days after the reading cannot be accurately determined; for example, after the injection of brilliant vital red II (table 14) the serum showed a distinct pink color on the 17th day after injection. Also a trace of this dye was excreted in the urine but the amount was too small for colorimetric reading. Following the large dose of brilliant vital red (new) the skin, mucous membranes and eye-lids became pink,

TABLE 14

*Decrease in color concentration with large dosage of certain dyes.
All readings in per cent*

TIME AFTER INJECTION	DOG				
	19-28 (20.13 pounds)	19-136 (14.5 pounds)	19-63 (52.5 pounds)	19-117 (42.5 pounds)	19-70 (53 pounds)
	Brilliant vital red II 20 mgm. per kilo	Brilliant vital red (new) 30 mgm. per kilo	T-1824 20 mgm. per kilo	T-1835 (alka- line) 20 mgm. per kilo	Trypan blue 40 mgm. per kilo
4 min.	71	70	72	71	
40 min.	63	61	67	67	
60 min.	61	58	65	62	
2 hrs.	55	50	60	55	49
4 hrs.	41	38	50	53	33
6 hrs.	38		45	49	24
8 hrs.	36	26.6	40	44	20
12 hrs.	30	19.3	29		15
2nd day	17.3	10.0	22	30.3	10
3rd day	11.0	4.4	12	15.0	7.6
4th day	5.3	2.1	10.2	12.0	5.6
5th day	2.0	1.4	8.2	9.0	4.6
6th day	1.2		5.0	6.3	3.2
7th day			4.0		
8th day			2.8		
9th day			1.6	3.8	
10th day					1.2

which color lasted only a few minutes. Months after injections of these vital red dyes we find traces of the dye in the mesenteric and other lymph glands which are stained a delicate pink.

The curve of dye elimination is incomplete for trypan blue because the large amount used (40 mgm. per kilo) was responsible for considerable hemolysis which obscured the readings for the first two hours. It is interesting to note the long period (10 days +) during which the dye is present in the blood serum.

Several dyes of group II were tested in the same way by the use of ten times the routine dose. In these experiments the rapid elimination of the dye from the blood is very striking.

Phenolsulphonephthalein (20 mgm. per kilo) shows a 4-minute blood serum reading 19 per cent and a fall to 4 per cent at the end of 40 minutes. Only 54 per cent of the dye was recovered in the urine during 24 hours and most of this during the first hour (dog 18-60). This same animal with a routine injection gave 72 per cent elimination in the urine.

Phenoltetrachlorophthalein (20 mgm. per kilo) shows a 4-minute blood serum reading of 7 per cent and a fall to 1.4 per cent at the end of 40 minutes. With smaller amounts of dye there is scarcely a trace of dye in the serum at the end of 4 minutes. With the large dose there was noted a mere trace of dye in the urine.

Buffalo fast crimson (20 mgm. per kilo) gives a higher color per cent for the 4-minute blood serum sample (35 per cent) but complete removal at the end of 40 minutes. After the large dose the urine contained 27 per cent of the dye but after 2 mgm. per kilo dye injection we note a dye elimination in the urine of 51 to 53 per cent.

Crystal S. scarlet (20 mgm. per kilo) gives a higher blood serum 4-minute reading (39 per cent) but also complete removal in 40 minutes. The urine contained only 7 per cent of the dye which was excreted almost completely during the first hour.

DISCUSSION

A summary of all dyes of group I appears in table 9. It is readily seen that there is a remarkable average for this group of standardized dogs tested by various groups of dyes. All groups give the same average figure of blood volume per kilo body weight as 92 to 93 cc. The speed of disappearance of dye from the blood stream is constant in the average for 40 minutes and reads 11 to 13 color per cent. This is really a striking uniformity in results and for this reason these figures cannot be put aside by any quibble about the action of colloids in the blood stream, adsorption phenomena, etc.

The outstanding fact remains as follows: Any one of a large series of dyes may be injected into the blood stream and within 2 minutes it is diluted to a certain color which remains almost unchanged for 4 to 6 minutes or longer. This indicates a circulating fluid volume of a certain bulk whatever the limiting structures of this area may be. We

cannot admit that a certain portion of the dye may escape from the blood capillaries or be phagocytocized during the first 2 minutes following the injection unless we admit the same or similar possibility for the second 2-minute interval. There is no such disappearance during this second period of 2 minutes, so no person without definite proof has the right to assume or postulate any such reaction during the first 2 minutes. If these facts established by these dye injections do not harmonize with our preconceived notions concerning blood volumes and fluids circulating in the living body, then it is time to modify these concepts.

SUMMARY

Blood volume measurement is not the property of a single dye substance or group of such substances but may be accomplished by a great number of dyes.

It is essential that the dye is non-toxic, and not stored in the tissues, that the colors are such as to permit accurate determination and that the dye is removed quite slowly from the blood stream. Given these characteristics a dye is suitable for blood volume work.

It is of some significance that all suitable dyes give a remarkably constant figure on the average for blood volume determinations (table 9). This may indicate that the fluid measured in the blood stream is relatively constant, whatever our mental reservations may be concerning the actual extent of this fluid medium or its limiting structures.

A blue azo dye (T-1824) in our hands is slightly superior to the vital red groups especially as regards ease and accuracy of colorimetric readings.

Several other dyes are studied and found to be satisfactory for routine blood volume work (table 5).

A great number of dyes are included in this study and are not suitable for blood volume work. Some are rapidly removed from the blood by way of the kidney or liver. The significance of these dye reactions cannot be discussed at this time.

BIBLIOGRAPHY

- (1) WHIPPLE, PEIGHTAL AND CLARK: Johns Hopkins Hosp. Bull., 1913, xxiv, 343.

BLOOD VOLUME STUDIES

IV. BLOOD VOLUME AS DETERMINED BY THE CHANGE IN REFRACTIVITY OF THE SERUM NON-PROTEIN FRACTION AFTER INJECTION OF CERTAIN COLLOIDS INTO THE CIRCULATION

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This investigation forms a part of the general program of this laboratory which includes a study of the complex problem of blood volume determinations. This problem is being approached from all angles and other publications dealing with the subject will appear in the near future. It occurred to us that certain colloids having a high refractive index might be introduced into the blood stream with a corresponding change in the refractive index of the serum. Acacia and gelatin are used and are found to appear quantitatively in the non-protein fraction of the serum. The change in refractive index of the non-protein fraction is sufficient to permit accurate determination of the degree of dilution affected by the mixture of the colloid solution with the circulating blood. This permits us to estimate with considerable accuracy the circulating blood volume by which these colloids are diluted and in which they circulate through the body. It is significant that blood volume as determined by this method compares very closely to the blood volume as determined by the vital red dyes. This indicates that the fluid areas included in the vascular system as measured by these various substances are of similar extent.

It is not our intention to review the many papers dealing with the different phases of blood volume work. We shall refer to the work of Keith, Rowntree and Geraghty (1), who first used the "vital red" method. We shall also give frequent references and figures which cover some of the work done in this laboratory by the use of "brilliant vital red."

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The recent work of Meek and Gasser (2) is of particular interest to us. These workers introduce intravenously large amounts of acacia, then remove some blood and make the proper analysis for the contained acacia. It is necessary to hydrolyze the acacia to liberate the pentosan which can then be determined quantitatively as a phloroglucid. One of us (Davis) carried out a number of experiments using this method but in our hands this method presented certain difficulties—it is very time-consuming and the figures are not at once available. The final gravimetric determinations are carried out on such small amounts of material that slight discrepancies in weighing cause large percentage errors. We feel that the method is unique and in theory valid.

The value of the refractometer in biological analysis has been especially emphasized by Reiss (3). This work has been somewhat elaborated in this country by Robertson (4). Although various workers have solved and suggested the attack of many problems in blood analysis, we have seen no reference to the application of refractometry to the determination of colloidal additions to the blood stream in blood volume work.

A substance to be suitable for injection in our work must possess certain qualities: *a*, It should go into solution in rather high concentration. *b*, It must be relatively non-toxic and non-antigenic. *c*, It must remain unchanged in the circulation at least until thoroughly mixed. *d*, It should have a high refractive index. *e*, It should be recoverable quantitatively in the non-protein fraction of the serum.

Among the substances considered, gelatin and gum acacia were finally chosen as best suited to our work. Proteins other than gelatin were considered, but were rejected for various reasons which are obvious. Starch and inulin disappear from the blood stream too quickly. Agar agar gels too readily, is hard to get into a concentrated solution and may produce grave intoxication as demonstrated on guinea pigs by Novy and De Kruif (5). Gum tragacanth will not form a concentrated solution, and is quite expensive. Possibly other substances colloidal in nature may be suggested by other investigators if this general method finds favor.

Gelatin and acacia are both relatively cheap and easily obtained; they may be made into 20 per cent to 25 per cent solutions with little difficulty; they are non-toxic and non-antigenic, as shown by Starin (6) and others for gelatin, and as shown by Gasser and Meek (2) and by De Kruif (7) for acacia; both remain in the serum non-protein fraction; both disappear from the blood stream quite slowly as Buglia (8) has shown for gelatin, and Meek and Gasser (2) for acacia.

The fluidity of concentrated solutions of acacia is of considerable advantage; equal concentrations of gelatin readily solidify upon cooling. On the other hand, gelatin has a much higher refractive index, thus permitting somewhat smaller injections than are used with acacia. Occasionally with acacia, never with gelatin, a tendency to vomit is noted in dogs, either during or shortly after injection. This nausea is of short duration and there are no observable bad after-effects. Usually such a reaction may be avoided by very slow injection (e.g., 5 cc. per minute).

TABLE 1
Refractive indices of gelatin and acacia solutions

REFRACTIVE INDICES OF 1 PER CENT ACACIA	REFRACTIVE INDICES OF 1 PER CENT GELATIN	MIXTURE EQUAL PARTS OF 1 PER CENT ACACIA AND 1 PER CENT GELATIN
0.00132	0.00168	0.00148
0.00124	0.00160	0.00144
0.00132	0.00172	0.00152
0.00136	0.00172	
Average 0.00131	0.00168	0.00148
REFRACTIVE INDICES OF 5 PER CENT ACACIA	REFRACTIVE INDICES OF 5 PER CENT GELATIN	MIXTURE EQUAL PARTS OF 5 PER CENT ACACIA AND 5 PER CENT GELATIN
0.00655	0.00837	0.00738
0.00663	0.00833	0.00742
0.00655	0.00837	0.00742
0.00662	0.00845	
Average 0.0065875	0.00838	0.0074066+
$0.0065875/5=0.0013175$	$0.00838/5=0.001676$	$0.0074066+/5=0.0014813+$

Since it is sometimes advantageous to know the refractive indices of the stock solutions with which we work, we have spent some time in determining the values for diluted specimens from such solutions, and of less concentrated solutions made from the dry material. We have used commercial gum acacia, obtained in small lumps or "tears," containing an appreciable amount of foreign particles; also "Gold Label" sheet gelatin. It may very well be that chemically purified materials give somewhat different refractive indices from those which we have recorded. It will be observed that the refractive indices of 1 per cent solutions of acacia are about 0.00131 or 0.00132 greater than the solvent-distilled water, and that of gelatin is about 0.00167 or

0.00168, whether the readings are made on 1 per cent solutions or on 5 per cent readings and divided by 5. We also have determinations on 2 per cent and 10 per cent solutions which are comparable. It may be said that the readings as noted in table 1 are on separate solutions in each case, not different readings on the same solution. Our percentage solutions are made by putting the dry (desiccated) substance in a volumetric flask and making up to volume. In case of concentrated solutions (above 10 per cent at least) suitable dilutions are made to facilitate reading. It will be noted that the mixtures of equal parts 1 per cent acacia and 1 per cent gelatin give a value (0.00148 +) very close to the theoretical mean (0.001495 +) as found in the other determinations cited.

METHOD

The Pulfrich refractometer which reads the angle of total reflection to within one minute has been used for all this work. A sodium flame is used as the source of light. By means of a table the refractive indices corresponding to angles of total reflection are determined directly. Ordinarily distilled water is used as a basis for comparison. For instance, the refractive index of a 1 per cent aqueous solution of sodium chloride is 0.00160 greater than that of distilled water. The angles of total reflection of both water and salt solution as read will depend upon the temperature, the refractive index of the refractometer prism, etc., but under the same conditions of reading this difference of 0.00160 remains constant. In principle our method depends upon the difference in reading between the non-protein fraction of the blood serum before and again after the injection of a solution of a suitable colloid into the circulation.

It will be observed that in many of our experiments we have used a concentrated solution of acacia plus gelatin; that is, a solution of 20 per cent to 25 per cent acacia and 10 to 20 per cent gelatin in Locke's solution or physiological salt solution. Such a mixture is filtered through cotton, or centrifugalized to get rid of foreign material. Upon standing or centrifugalization two rather definite layers appear. The upper layer forms a soft gel upon cooling, while the lower layer never gels, though viscid, and is easily pipetted from underneath the firmer top portion. This fluid mixture we employ for injections. It remains homogeneous and has a high refractive index, the 1 to 20 dilutions reading well over 0.00200. The top layer may be warmed and injected if so desired, though we have been accustomed to use it as part of the next stock batch of gelatin-acacia mixture made up.

We usually inject (in dogs) about 1 cc. of concentrated solution per pound body weight. This amount is usually measured directly into one or more syringes. Accurate measurement of this thick, viscid fluid presents certain difficulties. The solutions are so viscous that they do not rapidly drain from a pipette. We rinse the pipette with a little warm saline solution, or draw the fluid a certain distance beyond the mark and drain only a short while. If the latter method is employed, one should calibrate the pipette for delivery at a given temperature, and should allow a constant period for drainage. Again it may be advisable to calibrate a large syringe and draw the solution up directly. If desired the solution may be warmed after measurement in the cold by laying the syringe over a steaming dish or radiator with a rubber band over the opening to prevent loss of fluid when expansion takes place.

At least three tubes should be at hand for blood samples; two of these should be clean centrifuge tubes, or test tubes, for the serum samples; and one an hematocrit tube containing 2 cc. of 1.6 per cent sodium oxalate solution for the plasma sample. A second hematocrit tube may be prepared for a plasma sample after injection, if so desired.

In dogs the blood is ordinarily withdrawn from the external jugular vein. We take from 5 to 10 cc. into a dry tube for a serum sample, and 7 to 10 cc. into oxalate for a plasma sample before injecting. The injection should be made slowly, about 5 to 10 cc. per minute, the syringe rinsed with warm saline, and the washings injected. If the dog has veins easy to enter it is just as well to withdraw the needle after the injection is completed, and use the opposite side for the second blood sample. In case the veins are small and difficult to find, the needle may be kept open by slow injection of salt solution, and the second serum sample obtained on the same side. We allow 3 to 5 minutes after the injection for mixing before taking the second sample for serum.

After standing a few minutes, the clotted samples are detached from the sides of the tubes and centrifugalized for about 10 minutes for separation of the clear serum. We allow about 30 minutes at 2500 revolutions per minute for the plasma hematocrit samples.

In determining the non-protein fraction of the serum we follow the method as outlined by Robertson (4) in which the serum proteins are precipitated by N/25 acetic acid. Glass tubes 20 to 25 cm. long, having an inside diameter of at least 5 mm. and walls about 1 mm. thick, are sealed at one end. It is well to blow gently into the tube while the

sealed end is still soft, thus rounding the bottom and lessening the tendency to crack on cooling. These inexpensive tubes may be used over again after cleaning, until they become too short. Into these tubes, which have been carefully cleaned and dried, are introduced definite amounts of serum by means of any small bored pipette with a capillary tip. If possible, duplicates of each serum sample should be made. We use approximately 1 cc. amounts, although as little as 0.4 or 0.5 cc. is sufficient so long as exactly the same volume of N/25 acetic acid is added. In delivering the serum one should avoid wetting the upper part of the tube, and the formation of air bubbles.

The serum having been delivered, the same amount of N/25 acetic acid as of serum is introduced into each tube. The same pipette as used for measuring the serum, after being rinsed with distilled water and N/25 acetic acid, or a second pipette calibrated against it is used to measure the acid. In case acacia solution has been the injection fluid, the tubes of serum after injection will show much more cloudiness than the first sample, but this entirely disappears on subsequent shaking. The N/25 acetic acid solution may be made up with sufficient accuracy by diluting 4 cc. glacial acetic acid to 1750 cc.

A small glass bead is next dropped into each tube and the open end is sealed off in a flame, care being taken not to heat the contents. After cooling, the tubes are reversed a sufficient number of times to insure a thorough admixture of the contents. The tubes are next placed in a beaker containing cold water of such a depth as to completely immerse the tops of the fluid columns. It is well to pad the bottom of the beaker with cotton to guard against cracks in bumping when the water boils. The water is slowly heated to boiling and allowed to boil for 2 minutes. The tubes are then removed from the boiling water and cooled to room temperature, either in water or more slowly in air.

When the tubes have cooled, the sealed tips are broken off. The coagulum is broken up by means of a small clean wire, after which the tubes are centrifugalized for a few minutes. The clear supernatant fluid representing the non-protein fraction of the blood serum can be easily decanted or pipetted off. The samples taken before and those taken after injection are next read against each other in the refractometer. It is well to have a constant temperature in the room where the readings are done. To obtain this it may be necessary to leave the flame which is used as the source of light burning in the room for an hour or more before reading. Otherwise, at least in a long series of determinations, it may be necessary to re-read a given fluid such as

water or salt solution at frequent intervals to find the change due to rising temperature. Between separate samples the cup of the refractometer should be rinsed with distilled water and carefully dried with absorbent cotton or filter paper, preferably followed by lens paper.

We consider that the actual reading of the refractometer is the simplest step in the whole method. With a little practice anyone can bisect the opposite angles of the X with the well demarcated line between light and shadow shifted by the fine adjustment screen. It is better for one individual to make all the readings in a given experiment, since possible slight errors are then more likely to offset each other. The angles as read on the scale are referred to a table giving the corresponding refractive indices, and the difference between solutions is obtained at once by subtraction.

The plasma percentage before injection is calculated from the hematocrit tube readings, allowance being made for the oxalate solution previously in the tube. The total plasma withdrawn before injection is subtracted from the total fluid volume injected; the result we assume to be the increment to the plasma volume caused by injection.

A suitable dilution (e.g., 1 to 20) is made of the concentrated injection fluid and its increased refractivity over the salt solution solvent is determined.

We now calculate:

$$\frac{\text{Amount Injected} \times \text{Reading of Diluted Sample} \times \text{Dilution}}{\text{Difference in Refractivity of Non-Proteins}} = \text{Increased Plasma Volume}$$

$$\text{Plasma Volume Before Injection} = \text{Increased Plasma Volume} - \text{Plasma Increment.}$$

$$\text{Blood Volume} = \frac{\text{Plasma Volume (Before Injection)}}{\text{Plasma Per Cent}} \times 100.$$

The difference in refractivity of non-proteins is obviously twice the difference in refractometer readings, since each serum sample is diluted one-half with N/25 acetic acid.

In case simple solutions of gelatin or acacia are used the plasma volume may be determined as follows: Knowing the reading of a true 1 per cent solution, and that of an accurately diluted sample of the concentrated injection fluid, the number of grams injected may be determined. From the difference in the non-protein reading before and after the injection the concentration per cubic centimeter of plasma

can be calculated. Then the grams injected divided by the plasma concentration minus the plasma increment should give the same value as arrived at in the above formula. In case an hematocrit is taken after injection the above figuring may be done without subtracting the plasma increment and the larger result divided by the plasma per cent after injection; the result is probably somewhere near the blood volume after injection. From this the total volume injected minus the total blood withdrawn may be subtracted, and the resulting figure is often very close to the blood volume before injection as determined by the first formula. However, we consider the first method to be the more reliable.

In these calculations we are considering the vascular tree as a definite container with impermeable walls, into which a known fluid is injected, the whole mixed, and the total contents determined by the concentration of injected material in a given sample of the diluted fluid. Obviously we are not justified in these assumptions; indeed it is occasionally found that the plasma percentage in the sample after injection is less than it was beforehand. No doubt there are changes occurring in the blood stream that are quite beyond our control and about many of which we know nothing. There have been observations in this laboratory which indicate a frequent leucopenia after injection of acacia; sometimes there seems to be a definite delay of clotting after injection of acacia although *in vitro* it requires practically an equal volume of 10 per cent acacia to prevent clotting. However, other blood volume methods meet the same or similar difficulties, in that changes going on *in vivo* are not fully understood.

EXPERIMENTAL OBSERVATIONS

It must be demonstrated at once that acacia and gelatin alone or combined are not carried out of the blood plasma by blood coagulation. It must be shown that these two colloids may be recovered quantitatively from the non-protein fraction of the blood serum. Our experiments have convinced us that acacia and gelatin are not disturbed by blood coagulation and do appear quantitatively in the non-protein fraction of the blood when this method is employed as described. Experiment 1 may be taken as a typical example.

Experiment I. Recovery of acacia and gelatin in vitro. The following solutions were prepared:

1. One cubic centimeter 10 per cent acacia in NaCl solution + 9 cc. whole blood.

2. One cubic centimeter NaCl solution + 9 cc. whole blood. (Control.)
3. One cubic centimeter approximately 10 per cent gelatin in NaCl solution + 9 cc. whole blood.
4. One cubic centimeter NaCl solution + 1 cc. gelatin and acacia mixture in NaCl solution + 8 cc. whole blood.
5. Two cubic centimeters NaCl solution + 8 cc. whole blood. (Control.)
6. One cubic centimeter 10 per cent acacia in NaCl solution + 9 cc. NaCl solution.
7. One cubic centimeter approximately 10 per cent gelatin in NaCl solution + 9 cc. NaCl solution.
8. One cubic centimeter gelatin and acacia mixture in NaCl solution + 19 cc. NaCl solution.
9. NaCl solution.

Non-protein readings:

	<i>Angle of Ref.</i>	<i>Index</i>
1.....	$\begin{cases} 66^{\circ}6' \\ 66^{\circ}6' \end{cases}$	1.33855
2.....	$\begin{cases} 66^{\circ}26' \\ 66^{\circ}26' \end{cases}$	1.33695
3.....	$\begin{cases} 66^{\circ}4' \\ 66^{\circ}4' \end{cases}$	1.33871
4.....	$\begin{cases} 65^{\circ}50' \\ 65^{\circ}50' \end{cases}$	1.33983
5.....	$\begin{cases} 66^{\circ}26' \\ 66^{\circ}26' \end{cases}$	1.33695
Other solutions:		
6.....	$\begin{cases} 66^{\circ}4'30'' \\ 66^{\circ}4'30'' \end{cases}$	1.33867
7.....	$\begin{cases} 66^{\circ}3' \\ 66^{\circ}3' \end{cases}$	1.33879
8.....	$\begin{cases} 66^{\circ}6' \\ 66^{\circ}6' \end{cases}$	1.33855
9.....	$\begin{cases} 66^{\circ}21' \\ 66^{\circ}21' \end{cases}$	1.33735

Blood plasma percentage = 41.65 per cent.

$1.33855 (1) - 1.33695 (2) = 0.00160$; $0.00160 \times 2 = 0.00320$.

$0.00320 \times 0.4165 = 0.0013328$, reading of 1 per cent acacia in the whole blood.

$1.33867 (6) - 1.33735 (9) = 0.00132$, reading of 1 per cent acacia in NaCl solution.

$(100 \times 0.0013328 - 0.00132) / 0.00132 = 0.9$ per cent difference.

$$1.33871 (3) - 1.33695 (2) = 0.00176; 0.00176 \times 2 = 0.00352.$$

$0.00352 \times 0.4165 = 0.00146608$, 1 per cent gelatin in whole blood.

$$1.33879 (7) - 1.33735 (9) = 0.00144, \text{ 1 per cent gelatin in NaCl solution.}$$

$$(100 \times 0.00146608 - 0.00144)/0.00144 = 1.7 \text{ per cent difference.}$$

$$1.33983 (4) - 1.33695 (5) = 0.00288; 0.00288 \times 2 = 0.00576.$$

$0.00576 \times 0.4165 = 0.00239904$, reading of 1:10 dilution of gelatin and acacia mixture in blood.

$$1.33855 (8) - 1.33735 (9) = 0.00120, \text{ reading of 1:20 dilution of mixture.}$$

$2 \times 0.00120 = 0.00240$, reading of 1:10 dilution of gelatin and acacia mixture in NaCl solution.

$$(100 \times 0.00240 - 0.00239904)/0.00240 = 0.04 \text{ per cent difference.}$$

It will be seen that within experimental error the recovery of acacia and gelatin was complete in whole blood. Where the gelatin and acacia were used separately a slight positive error occurred, and when a mixture of the two was employed the check was almost perfect.

For a considerable range of angles the index per minute is 8 in the 5th decimal place; hence the difference between two solutions is the difference in minutes $\times 0.00008$. As:

$$66^{\circ} 26' - 66^{\circ} 6' = 20'; 20 \times 0.00008 = 0.00160$$

In ordinary calculations we seldom use decimals in referring to refractive indices, thus for convenience we generally call that of acacia simply 132, and gelatin 168, etc.

It has been of interest to determine whether the method can be used with accuracy in the presence of hemolysis, jaundice, lipemia, etc. These conditions sometimes offer considerable difficulties in dye determinations of blood volume. We have found that these factors introduce no error in the refractometric method. Hemoglobin is entirely precipitated with the proteins. We have never taken any special precautions to prevent hemolysis, consequently a moderate grade has sometimes occurred. Such experiments have always been tabulated as normal, without question. Bile pigments and fat even in excessive amounts are inconsequential factors after the processes of dilution and precipitation.

The following experiment illustrates recovery in the presence of bile.

Experiment II. Recovery of media in the presence of bile. Sample: 100 cc. fresh blood + 5 cc. dog's bile.

The following solutions were prepared:

1. Nine cubic centimeters serum (containing bile) + 1 cc. 0.9 per cent NaCl solution.

2. Nine cubic centimeters serum (containing bile) + 1 cc. gelatin and acacia mixture in 0.9 per cent NaCl solution—(3 samples).

3. Nine cubic centimeters 0.9 per cent NaCl solution + 1 cc. gelatin + acacia mixture (2 samples).

4. Nine hundredths per cent NaCl solution.

Non-protein readings:

1.....	$\begin{array}{l} \text{Angle of Ref.} \\ \left\{ \begin{array}{l} 66^{\circ}38'30'' \\ 66^{\circ}38' \end{array} \right. \end{array}$	Average $66^{\circ}38\frac{1}{4}'$
2.....	$\begin{array}{l} \left\{ \begin{array}{l} 66^{\circ}11'30'' \\ 66^{\circ}11'30'' \\ 66^{\circ}11' \end{array} \right. \end{array}$	Average $66^{\circ}11\frac{1}{4}'$
Difference (1 and 2) = $26\frac{1}{2}'$; $26\frac{1}{2} \times 8 \times 2 = 430\frac{2}{3}$.		
Readings of media:		

3.....	$\begin{array}{l} \text{Angle of Ref.} \\ \left\{ \begin{array}{l} 65^{\circ}27' \\ 65^{\circ}26'30'' \end{array} \right. \end{array}$	Average $65^{\circ}26\frac{1}{4}'$
4.....	$66^{\circ}20'$	
Difference (3 and 4) = $53\frac{1}{4}'$; $53\frac{1}{4} \times 8 = 426$.		
$100 \times 430\frac{2}{3} - 426 / 426 = 1.09$ per cent error.		

It will be seen at once that the difference in recovery in NaCl solution and in jaundiced serum lies well within the experimental error of measurement and reading, and that even so, the error as noted is in favor of the serum mixture.

The following two experiments were performed upon the same dog, one during an induced lipemia, the other in a normal period. We have another experiment on the same animal on still another date which gives practically the same blood volume which these experiments show.

Experiment III. Blood volume in lipemia. August 13, 1919.

Dog 20-6. Young male adult, in good condition. Weight $23\frac{1}{2}$ pounds (10.52 kgm.). Gave 50 cc. cottonseed oil + 100 cc. milk by stomach tube $1\frac{1}{2}$ hours before withdrawal of blood.

Removed 16 cc. blood before injection. Plasma per cent = 57.

Injected $24\frac{1}{2}$ cc. media + 20 cc. NaCl solution. Removed 18 cc. after 6 to 7 minutes.

Serum shows marked lipemia.

Media diluted 1:20 — reading $66^{\circ}2'$18' difference

NaCl solution — reading $66^{\circ}20'$ $18 \times 8 = 144$

Serum non-protein, before injection — reading..... $\left\{ \begin{array}{l} 66^{\circ}19\frac{3}{4}' \\ 66^{\circ}19\frac{3}{4}' \end{array} \right.$

Serum non-protein after injection — reading..... $\left\{ \begin{array}{l} 66^{\circ}13\frac{1}{2}' \\ 66^{\circ}13\frac{1}{2}' \end{array} \right.$

Difference = $6\frac{1}{4}'$; $6\frac{1}{4} \times 8 \times 2 = 100$.

44.5 cc. total injection

9.0 cc. plasma withdrawn

35.5 cc. plasma increment

$$\frac{144 \times 24\frac{1}{2} \times 20}{100} - 35.5 = 670 \text{ cc. plasma volume.}$$

$670 / 0.57 = 1176 \text{ cc. blood volume.}$

$1176 / 10520 = 11.1 \text{ cc. per 100 grams body weight.}$

Experiment IV. August 27, 1919.

Dog 20-6. Young male adult (see exper. III). Weight 24 pounds (10.9 kgm.).

Removed 16 cc. blood before injection. Plasma = 54.7 per cent.

Injected 25 cc. acacia + gelatin mixture (previously found to read 164 in a 1:25 dilution) + 14 cc. NaCl solution.

Dog became a little sick but did not vomit.

Second blood sample withdrawn 4 - 5 minutes after injection.

1. Non-protein—before injection..... $\left. \begin{array}{l} \text{Angle of Ref.} \\ 66^{\circ}19'30'' \\ 66^{\circ}19'30'' \\ 66^{\circ}20' \end{array} \right\}$

2. Non-protein—after injection..... $\left. \begin{array}{l} 66^{\circ}11' \\ 66^{\circ}11' \\ 66^{\circ}11' + \\ 66^{\circ}11' + \end{array} \right\}$

Angle of Ref.

No. 1 (before injection) re-read because of rising temperature.. $66^{\circ}21'$
Difference about $9\frac{1}{2}$ ($66^{\circ}20\frac{1}{2}' - 66^{\circ}11'$)

$$9\frac{1}{2} \times 8 \times 2 = 152$$

39 cc. total injection — 9 cc. plasma withdrawn = 30 cc. plasma increment.

$$\frac{164 \times 25 \times 25}{152} - 30 = 674 \text{ cc. plasma volume.}$$

$674 / 0.547 = 1177 \text{ cc. blood volume.}$

$1177 / 10900 = 10.7 \text{ cc. per 100 grams body weight.}$

That the blood volumes as determined in experiments III and IV so nearly coincide is a mere chance, especially when the time interval between determinations, the change in weight, etc., are considered. Nevertheless, the results certainly suggest that even a marked lipemia does not affect the non-protein readings.

With the possibility of error from these more or less abnormal conditions eliminated, we may go on to the consideration of normal cases.

Table 2 gives the results of blood volume determinations in a number of dogs. It is scarcely necessary to give protocols of the individual experiments; the essential details are given in the table, and experiments III and IV are indicative of the usual procedure and calculations.

Most of these dogs were in good condition, a few were very anemic as noted in the remarks.

In nearly every case we have dye determinations on the animal; some of these were done in the regular course of experiments by various laboratory workers, others were kindly performed by either Mr. H. P. Smith or Mrs. Robscheit, especially to serve as checks for our own experiments.

Blood volumes done by the dye method at an interval of several days or weeks from the refractometric method are of little comparative worth; the determinations done on the same day are of the greatest value. Sometimes the difference between the two results is rather large, but on the other hand, the similarity is often quite striking. We were pleased to find that the refractometric method sometimes gives higher results, sometimes lower than the dye method. This lack of constancy probably indicates the occurrence of experimental errors rather than a fundamental fault in the method. The ratio of blood volume to body weight is quite similar in the two methods. The average amount of blood per 100 grams body weight in the twenty-one experiments tabulated in table 2 is 9.76 cc.

In a series of dogs reported by Meek and Gasser (2) using the acacia-phloro-glucid method, the blood volume in per cent of body weight was 9.74 per cent. To compare with this, our results as given should be multiplied by the specific gravity of blood, but even so the similarity is rather striking. The plasma percentages of body weight in dogs as determined by Keith, Rowntree and Geraghty (1) are quite comparable to our results.

Reference to table 2 shows that we gave much larger injections in the early experiments than we did later. The later work was largely done with the solutions of high refractive indices. Furthermore, we came to the conclusion that large volumes of fairly concentrated colloids produced greater upset of equilibrium (i.e., changes in cell content and ratio, unaccountable plasma percentages, disturbances in protein content, etc.) than did small injections, and were to be avoided when possible. We attempted to obtain the blood volume in a few instances when Mr. Smith and Mr. Arnold injected very large amounts of 6 per cent acacia without previously producing an anemia. In two experi-

TABLE 2
Blood volume results—refractometric method—dogs

EXPERIMENT NUMBER	DOG NUMBER	DATE	WEIGHT	INJECTION	PLASMA BEFORE INJECTION	PLASMA VOLUME	BLOOD VOLUME	BLOOD PER 100 GRAMS BODY WEIGHT	BLOOD VOLUME DYE METHOD	REMARKS
			<i>kgs.</i>		<i>per cent</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>		
V	18-114	7/25/18	11.64	60 cc. 20 per cent acacia	49	611	1247	10.7		See next experiment
VI	18-114	8/3/18	11.50	60 cc. 20 per cent acacia	44.4	547	1232	10.7	1294	Dye determination made August 14
VII	18-113	7/25/18	12.44	60 cc. 20 per cent acacia	46.8	491	1049	8.4	940 (1) 1136 (2)	Dye determinations made (1) June 10, and (2) August 14
VIII	18-115	8/1/18	7	50 cc. 20 per cent acacia	60.58	461	760	10.8	761 (1) 1000 (2)	Dog is in poor condition. Dye determinations (1) June 10, and (2) September 19
IX	18-24	3/12/19	13.6	54½ cc. 20 per cent acacia		582	1294	9.5	1305	Calculated volume after injection. Dye determination also after injection
X	18-39	3/14/19	11.1	45 cc. 20 per cent acacia		553	1270	11.4	1255 (1) 1205 (2)	Calculated volume after injection Dye determinations (1) before and (2) after injection
XI	18-4	3/18/19	20.5	86 cc. 20 per cent acacia	37.1	804	2167	10.5	2264 (1) 2117 (2)	Dye determinations (1) before and (2) after injection of acacia

	19-11	4/15/19	20	64 cc. 25 per cent acacia	47	854	1817	9.1	1955 (1) 2630 (2)	Dye determinations (1) before and (2) after injection of acacia
XII										
XIII	17-157	8/8/19	10.02	34½ cc. 20 per cent acacia	50	514	1028	10.2	1123	Dye determination made on a different day, same week
XIV	18-38	7/7/19	10.68	34 cc. mixture gel- atin and acacia	48.21	464	962	9.0	903	Dye injected in acacia solution
XV	18-38	8/4/19	10.45	25 cc. mixture gelatin + acacia	50.8	513	1009	9.5		See previous experi- ment
XVI	16-160	8/9/19	12.045	24½ cc. mixture gelatin + acacia	45.2	540	1323	10.9	1040	Dye determination made August 18
XVII	18-126	8/12/19	10.795	24½ cc. approxi- mately 16 per cent gelatin	46.9	556	1185	10.9	975	Dye determination made August 18
XVIII	19-93	8/22/19	12.727	25 cc. mixture gel- atin + acacia	68.5	795	1160	9.1	1023	Dog anemic. Dye de- termination made on previous day
XIX	19-94	8/22/19	13.80	25 cc. mixture gel- atin + acacia	70	909	1298	9.4	1090	Dog anemic. Dye de- termination made on previous day
XX	19-95	8/23/19	13.98	25½ cc. mixture gelatin + acacia	63	718	1139	8.1	1106	Dog anemic. Dye de- termination made two days previously
XXI	19-96	8/23/19	14.204	25½ cc. mixture gelatin + acacia	61.2	642	1049	7.3	993	Dog anemic. Dye de- termination made two days previously

ments out of about half a dozen we obtained results comparable with the dye method. Such enormous injections are certainly unnecessary and unfavorable for subsequent refractometric determinations. Experiment XXVI shows one of the few good results which we obtained.

Experiment XXVI. Blood volume determined following the injection of a large amount of 6 per cent acacia solution. August 8, 1919.

Dog 17-38. Weight 28 pounds (12.72 kgm.).

Mr. Arnold injected 635 cc. of approximately 6 per cent acacia; this was the estimated plasma volume.

Sample following injection was removed in 5 minutes.

A 1:6 dilution of the injection media read 120.

Plasma per cent = 42.5 immediately preceding injection.

Difference in non-protein readings before and after = $22\frac{1}{2}; 22\frac{1}{2} \times 8 \times 2 = 360$.

$$\frac{120 \times 6 \times 635}{360} - 635 = 635 \text{ plasma volume before injection.}$$

$635 / 0.425 = 1496$ cc. blood volume.

Mr. Arnold obtained the following results by the dye method:

	Plasma volume	Blood volume
Before injection.....	585	1416
After injection.....	901	1567

In addition to experiments on dogs we have a few observations on rabbits.

With these animals we use an improvised box holder with side holes through which the ears are drawn. Blood is obtained from the marginal ear vein through a small longitudinal slit made with a safety razor blade. Collection is made into small tubes, one for plasma and one for serum, as with dogs. Three cubic centimeter hematocrit tubes are made from sections of straight-sided, graduated 10 cc. pipettes; one end of a section is sealed off, and the graduations subsequently verified or modified if necessary. To prevent clotting 0.4 or 0.5 cc. of 1.6 per cent oxalate solution is used per tube. To dilate vessels and hasten bleeding an electric light bulb held almost touching the ear is found very satisfactory. Injection of the colloidal material is made into any available ear vein from a syringe with a small needle, and collection of blood sample after injection made from the marginal vein of the opposite ear. Using this technique little blood need be taken, and many determinations may be made upon the same animal with minimal injury. Table 3 gives the results on four rabbits.

The amounts of solution injected have always been quite small and no untoward reactions have been noted. The checks with dye determinations differ by 4.8 per cent to 11 per cent.

TABLE 3
Blood volume results on rabbits—refractometric method

EXPERIMENT NUMBER	RABBIT NUMBER	DATE	WEIGHT	INJECTION	PLASMA BEFORE INJECTION	PLASMA VOLUME	BLOOD VOLUME	BLOOD PER 100 GRAMS BODY WEIGHT	BLOOD VOLUME DYE METHOD	REMARKS
XXVII	I	7/23/19	grams 3375	10 cc. 20 per cent acacia	per cent 60	cc. 149	cc. 248	grams 7.3	260	Samples removed during period 8 to 17 minutes after injection. Dye de-termination made on same day
XXVIII	I	7/29/19	3375	9.5 cc. 20 per cent gelatin	64.8	159	245	7.2		Sample removed 10 minutes after in-jection
XXIX	I	8/7/19	3300	5 cc. concentrated mixture gelatin + acacia + 5 cc. 0.9 per cent NaCl solution	60.5	125	206	6.2		Sample removed 5 minutes after in-jection
XXX	I	8/24/19	3385	5 cc. concentrated mixture gelatin + acacia + 5 cc. 0.9 per cent NaCl solution	62.7	145	231	6.8		Sample removed 5 minutes after in-jection
XXXI	II	7/24/19	3100	10 cc. mixture gel- atin + acacia	65.66	154	250	8.06	220 (1) 226 (2)	Sample removed 30 minutes after in-jection. Dye de-terminations made on same day (1) with ear veins (2) femoral vein and artery

[illegible]

The large ratio of blood volume to weight is rather interesting. We think that without doubt the blood volume as determined by samples obtained 5 minutes after injection should be considered the most accurate. Averaging the values obtained from these 5-minute samples we find that the average of 7.07 cc. of blood per 100 grams body weight is reduced to 6.49. Even this is much higher than the values usually accepted. Meek and Gasser (2) give an average of 5.44 per cent of the body weight, and other observers even less.

We have several experiments on rabbits where blood samples were withdrawn at varying intervals, which show increasing blood volumes as time goes on. This, of course, is due to at least two factors: *a*, the actual elimination of acacia, and *b*, the dilution of the plasma, presumably by fluid drawn into the blood stream from the tissues because of increased osmotic pressure. The following abbreviated protocol of experiment XXXV shows the changes mentioned.

Experiment XXXV. Blood volume. August 15, 1919.

Rabbit III. Weight 3175 grams.

Injected 10 cc. gelatin + acacia mixture (reading 1:12½ dilution = 0.00172) and 3 cc. of 0.9 per cent solution NaCl.

I. Sample before injection. Plasma per cent = 59. (Removed 4.35 cc. blood). Non-protein readings $\begin{cases} 66^{\circ}25' \\ 66^{\circ}25' \end{cases}$

II. Sample removed 5 minutes after injection. Non-protein readings $\begin{cases} 66^{\circ}15\frac{1}{2}' \\ 66^{\circ}15\frac{1}{2}' \end{cases}$

III. Sample removed 15 minutes after injection. Non-protein readings $\begin{cases} 66^{\circ}16' \\ 66^{\circ}16' \end{cases}$

IV. Sample removed 30 minutes after injection. Plasma per cent = 61. Non-protein readings $\begin{cases} 66^{\circ}17\frac{1}{2}' \\ 66^{\circ}17\frac{1}{2}' \end{cases}$

V. Sample removed 1 hour after injection. Plasma per cent = 63. Non-protein readings $\begin{cases} 66^{\circ}18\frac{1}{2}' \\ 66^{\circ}18\frac{1}{2}' \end{cases}$

VI. Sample removed 20 hours after injection. Plasma per cent = 61.7. Non-protein readings $\begin{cases} 66^{\circ}22' \\ 66^{\circ}22' \end{cases}$

Altogether, 23.27 cc. blood were removed in 10 samples.

Calculating as in previous experiments the results are approximately:

SAMPLE	PLASMA VOLUME	BLOOD VOLUME	PER 100 GRAMS BODY WEIGHT
5 minutes.....	118	200	6.3
15 minutes.....	124	210	6.6
30 minutes.....	148	241	7.5
1 hour.....	169	268	8.4

It will be noted that the plasma percentage increased for at least an hour. Such variations in values with lapse of time inclines us to accept the results obtained from 5-minute samples. This probably gives enough time for mixing and a minimal time for elimination and dilution: The reading of the sample after 20 hours indicates that an appreciable amount of injection media is still in the circulation. We have numerous observations of a similar nature showing that total elimination is slow, and corroborating the results of previous workers.

We have done very little work with cats because of a scarcity of animals. Our technique has been to inject into the femoral vein, and withdraw blood from the femoral artery through a cannula. Our experiments are so few in number that we consider it unwise to report these experiments at this time.

DISCUSSION

The colloid-refractometric blood volume method as outlined gives results quite comparable to the dye methods, and to Meek and Gasser's acacia method. This method requires a slightly longer time to complete than the dye method but it is much shorter than the method of Meek and Gasser. A routine determination can be finished in about two hours.

We have considered reading the plasma or serum difference without precipitating the proteins, but the dilution of the protein content makes such determination altogether unreliable. An alternate possibility, however, is to use the plasma non-proteins, which necessitates only one blood sample before injection and one afterward. A serious drawback to such a procedure is that the samples put into oxalate solution must be measured and of exactly equal volume in order that one may be read against the other; also the dilution with oxalate must be considered in computations. However, this method can be employed in conditions in which the blood for any reason fails to clot or clots incompletely.

We have tried double blood volume determinations, that is, on the basis of two separate injections at short intervals, only twice (on rabbits) and then without success. The recovery after the second injection was less than was to be expected in these instances. We can offer no explanation of this failure; probably further work with dogs as well as rabbits will clear up the difficulty.

If Meek and Gasser (2) can recover acacia quantitatively as phloroglucid after a second injection, presumably there should be no difficulty in recognizing it refractometrically.

It has occurred to us that the transient white cloudy precipitate formed upon adding dilute acetic acid to blood serum containing acacia might be used as a qualitative method in studying the elimination curve of acacia from the blood stream.

The injection media and materials used in this method are comparatively cheap and are always available. To laboratories equipped with a refractometer we offer the procedure as worthy of trial. The time needed for a complete determination is not too long for ordinary work, the individual steps are relatively simple, and the results compare favorably with those obtained by other methods. A point of considerable advantage is the small amount of blood needed for the determinations; if necessary, accurate results should be obtained with one cubic centimeter of serum before, and one after injection, and a micro-hematocrit reading. Another point of value in the method is that hemolysis, lipemia and cholemia are not disturbing factors.

SUMMARY

We have outlined a method of determining blood volume which consists essentially in reading refractometrically the serum non-protein increase after the intravenous injection of a known amount of acacia or gelatin solution, or a mixture of the two.

By this method we have found in the dog an average of 9.76 cc. blood per 100 grams body weight. In the rabbit an average of determinations using samples removed 5 minutes after injection of media gives a volume of 6.49 cc. per 100 grams body weight.

Of great importance is the fact that hemolysis, lipemia and cholemia do not affect the accuracy of determinations by this method.

BIBLIOGRAPHY

- (1) KEITH, ROWNTREE AND GERAGHTY: *Arch. Int. Med.*, 1915, xvi, 547.
- (2) MEEK AND GASSER: *This Journal*, 1918, xlvii, 302.
- (3) REISS: *Beitr. z. chem. Physiol. u. Path.*, 1903, iv, 150; *Arch. f. exper. Path. u. Pharm.*, 1903, li, 18; *Ergebn. der inneren Med. u. Kinderheilkunde*, 1913, x, 531; *Zentralbl. Biochem. Biophysics*, 1913, xv, 181; *Zentralbl. Biochem. Biophysics*, 1915, xviii, 273; *Deutsch. Arch. Klin. Med.*, 1915, cxvii, 175.
- (4) ROBERTSON: *Journ. Biol. Chem.*, 1912, xi, 179; 1912-13, xiii, 325; 1915, xxii, 233.
- (5) NOVY AND DE KRUIF: *Journ. Infec. Dis.*, 1917, xx, 537.
- (6) STARIN: *Journ. Infec. Dis.*, 1918, xxiii, 139.
- (7) DE KRUIF: *Ann. Surg.*, 1919, lxix, 297.
- (8) BUGLIA: *Biochem. Zeitschr.*, 1909-10, xxiii, 215.

A COMPARATIVE STUDY OF HEMOGLOBIN DETERMINATION BY VARIOUS METHODS.

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The regeneration of hemoglobin and red cells following simple anemia has been studied in this laboratory for more than 2 years. A preliminary report of this work by Whipple and Hooper (13) has appeared and it is obvious that this curve of hemoglobin regeneration can be influenced by a number of diet factors. In this work it is essential that there be an accurate determination of hemoglobin. For this reason a comparative study of many hemoglobin methods was undertaken. It is believed that the method finally adopted for this work will be of value to other workers in the experimental field as well as to hospital and school laboratories where routine hemoglobin readings are so frequent. It need not be stated that much of the work expended upon routine hemoglobin determination is a total loss because the instrument used has not been standardized or the method is inaccurate. Too little is known as to the normal hemoglobin value in human beings as affected by age, altitude, climate, etc. No comparison is possible until some accurate standard is adopted for the routine work.

The earlier anemia work in this laboratory was done with Sahli's method using his modification of Gowers' instrument. It was soon apparent that incorrect results were being obtained and that the hemoglobin percentages were considerably higher than they should be when correlated with the other blood findings. The standard color tubes when checked against the oxygen capacity method of Van Slyke (12) showed great variations in color density. New tubes were purchased and when standardized showed much fading varying from 5 to 20 per cent. The

results obtained with these tubes were therefore sufficiently erroneous to warrant the discarding of this method for hemoglobin determination.

The Palmer carbon monoxide method was next tested and accurate results were obtained, provided the standard solutions were frequently checked.

Since the publication of Newcomer's method based on spectrophotometric data this method has also been carefully investigated, as well as a combination of Palmer's and Sahli's method with slight modifications described below.

History of Methods.

I. Acid Hematin Method.—Sahli (10), finding that methods employing artificial color standards were not satisfactory, brought forth the acid hematin method. Hemoglobin is converted into acid hematin by the addition of 0.1 *N* HCl and then compared with a standard of like material. Numerous criticisms of the method have appeared and as many modifications been offered. Berzeller (2) claimed that the presence of lipoids alters readings. Stäubli (11) called attention to the time factor for maximum color development. Palmer (9) claimed that the standard is not permanent, that there is considerable delay in maximum color development, and that the method is not applicable for blood of different species. Haessler and Newcomer (4) offered a modification in the instrument used, using eleven standard tubes of different concentrations for comparison. Lilliendahl-Petersen (7) employed Sahli's principle in a Tallquist form. Newcomer (8) recently published a method of hemoglobin determination by comparing an acid hematin suspension of blood with a piece of brown-colored glass of definite thickness. The method is based on spectrophotometric data. The comparison is made with a Duboscq type of colorimeter.

II. Carbon Monoxide Method.—Hoppe-Seyler in 1892 (6) published his procedure of accurately determining hemoglobin in the form of carbon monoxide hemoglobin. The technical difficulties involved were too numerous for general adoption of the method.

Haldane (5) 8 years later revived Hoppe-Seyler's principle of hemoglobin determination but in a much simpler form. He used Gowers' instrument.

Palmer (9) in 1918 published a method which has found much favor. The principle is that of Hoppe-Seyler's procedure; *i.e.*, a color comparison of carbon monoxide hemoglobin solution with a standard of known hemoglobin content. Ammonia solution is used as a diluent instead of water. The color comparison is made in a Duboseq colorimeter.

EXPERIMENTAL.

I. Palmer Method.—We have used Palmer's method with only slight modifications. All experiments carried on during this investigation have been done on dogs. All blood is obtained by venous puncture. About 10 cc. of blood are drawn from the jugular vein with a glass syringe and emptied into a graduated centrifuge tube containing 2 cc. of a 1.6 per cent sodium oxalate solution. The plasma obtained by centrifugalization is carefully pipetted off, the tube slightly tilted, and a 1 cc. calibrated pipette of small lumen quickly inserted with the finger closing the opening at the upper end. Blood is slowly drawn up to the 1 cc. mark, the pipette is thoroughly wiped on the outside, and its contents are transferred into a small test-tube. The pipette is carefully rinsed in 2 cc. of N salt solution previously measured with this same pipette and emptied into a test-tube similar to the one containing the blood. This salt solution, now containing some red cells, is then carefully added to the 1 cc. of blood and the whole thoroughly mixed avoiding of course too vigorous shaking. Extreme care must be taken with this procedure so that the suspension of packed red blood cells is truly a dilution of one in three. The latter was ascertained to be the most convenient dilution, for the hemoglobin of our normal dogs is usually considerably over 100 per cent, frequently showing readings of 130 to 140 per cent. From the diluted blood cells suspension a 1 per cent solution of blood is made. 1 cc. of the diluted blood, 1:3, is drawn up into the same pipette used for diluting the packed cells as well as for measuring the original amount, is transferred to a 100 cc. volumetric flask containing

the 0.4 per cent ammonia solution, and made up to the 100 cc. mark with this same diluent. The solution is thoroughly mixed and at once saturated with carbon monoxide and read immediately. The percentage of hemoglobin obtained is multiplied by three—the packed red cells having been previously diluted 1:3—and this figure again multiplied by the red cell percentage of the blood computed from the hematocrit readings. In order to determine the accuracy of this apparently roundabout procedure, which is employed to prevent further bleeding for hemoglobin determination, readings were made from whole blood collected from the vein directly into a vessel containing sufficient dry sodium oxalate to prevent clotting and compared with the figures

TABLE I.*

Hemoglobin of packed red blood cells diluted 1:3.	Undiluted packed red cells.	Red cells from hematocrit.	Estimated hemoglobin from packed cells.	Hemoglobin of whole blood direct.	Difference.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
69	207	46.0	95	94	+1
57	171	48.0	82	83	-1
67	201	40.0	80	80	0
59	177	47.2	84	84	0
62.1	186.3	50.5	94	94	0
76.9	230.7	42.6	98	97	+1
63.0	189	38.4	73	73	0

* This work has been repeated and similar results have been obtained.

obtained by our means. Table I demonstrates the accuracy of the method as we employ it. It is obvious that by increasing the amount of hemoglobin used we tend to diminish any error of the method. 1 cc. of packed red cells should give more accurate readings than 20 mm. of whole blood.

Palmer's technique for the preparation of his standards has been closely adhered to. Palmer used either defibrinated ox or human blood. We have tried whole as well as defibrinated blood. Furthermore, blood obtained from different species has been investigated. In some cases blood has been obtained aseptically and the standard prepared from it with all aseptic precautions. With other standards only the usual cleanliness has been exercised.

The figures in Table II demonstrate the amount of fading of standards during the time of observation.

TABLE II.
Change in Color Value of Palmer Standards of Hemoglobin.

Standard No.	Source of blood.	Aug. 8, 1918.	Aug. 23, 1918.	Sept. 20, 1918.	Oct. 21, 1918.	Nov. 15, 1918.	Dec. 24, 1918.	Jan. 24, 1919.	Mar. 5, 1919.	April 1, 1919.	May 16, 1919.	June 23, 1919.	Aug. 5, 1919.	Sept. 7, 1919.	Oct. 21, 1919.	Total fading.	Remarks.
I	Dog (whole).	100	99	90	75	73	74	74	74	72	71					29	Discarded. Slight brownish tinge last 3 months.
II	Goat " sterile.		100	85	80	73	74	74	75	75	76	75	75	75		26	Discarded. Slight brownish tinge last 3 months.
III	Dog (defibrinated).			100	99	89	87	86	86	80	80	81	82	82	82	20	No color change.
IV	Human "					100	93	89	89	84	84	84	85	85	84	16	" "
V	Sheep "							100	98	91	91	92	90	90	90	10	" "
VI	Dog (whole).								100	93	88	87	85	86		15	Spoiled.
VII	" (defibrinated).									100	100	100	100	100	100	0	No color change.
VIII	" "										100	99	99	96	95	5	" "

Discussion of Table II.

No. I showed the greatest fading, 29 per cent in 8 months, the maximum change taking place during the first 2 months. The slight color change did not interfere with color comparison.

No. II was prepared from goat blood with aseptic precautions and kept sterile throughout the experiment. Neither the fragility of red cells nor bacterial decomposition seems to play an important rôle in the fading of color. The change was nearly as much as with No. I, the maximum fading taking place during the first 3 months.

The curve of No. III is more promising, defibrinated blood showing slightly better keeping qualities than whole blood. The fading was not so rapid nor so much as in Standards I and II, nor was any color change apparent.

No. IV prepared from pooled defibrinated human blood showed about the same stability of color as defibrinated dog blood. The greatest change took place during the first 2 months; after that the fading was slight.

■ We were more successful in keeping No. V, prepared from defibrinated sheep blood, for the maximum fading was but 10 per cent as compared with 16 and 20 per cent when using defibrinated human and dog blood.

No. VI, having as its source whole dog blood, faded 15 per cent during 6 months, not so much as Standards I and II also prepared from whole blood, but still slightly more than when the blood was previously defibrinated.

Standard VII again prepared from defibrinated dog blood was the only one which remained unchanged during the period of observation, 7 months.

Standard VIII, for which defibrinated dog blood was again employed, showed a fading of 5 per cent during 5 months. While more promising than the earlier ones, still the change is too much for accurate hemoglobin determinations.

Another standard prepared from whole dog blood to determine again the difference obtained with whole and defibrinated blood is but 2 months old. The fading already amounts to 7 per cent during this period.

II. Acid Hematin Method.—Because of the uncertain stability of carbon monoxide hemoglobin solutions we have attempted to solve the problem by searching for a more stable color standard. In view of Sahli's work acid hematin was tried again. It offers an easier color comparison than do the reds of oxyhemoglobin and carbon monoxide hemoglobin. Sahli in his original work obtained satisfactory results, as did several of his coworkers. We have combined Palmer's method with Sahli's principle, that is, determined hemoglobin in the form of acid hematin, employing Palmer's method of standardization. The sealed tubes containing dilute acid hematin suspension as purchased (Sahli instrument) are unsatisfactory as was pointed out in the history of the methods. The thought that acid hematin in a more concentrated form might not fade so readily arose, and therefore 5, 10, and 20 per cent suspensions were investigated. Our method of procedure was as follows:

The oxygen capacity of a sample of dog's blood was determined by Van Slyke's method and the hemoglobin content computed therefrom. An acid hematin standard in the form of a 20 per cent suspension, so diluted that a 1 per cent dilution prepared from it would read 100 per cent, was made. Because of our experience with whole and defibrinated blood in the preparation of standards for the carbon monoxide method we employed defibrinated blood for our first standard. It is well known and has been mentioned in the history of acid hematin methods that the time factor for allowing the maximum color of acid hematin to develop plays a very important rôle. This standard, after preparation and dilution, was allowed to stand 24 hours to insure correct readings. A 1 per cent dilution prepared from the 20 per cent suspension was of course employed for direct color comparison. The same time factor was used for the blood to be tested. A 1 per cent suspension of blood is used for the determination of hemoglobin. 0.1 N HCl is employed throughout the procedure, for the original standard suspensions as well as for all further dilutions. The strength of HCl used within a certain limit is immaterial. We tried N, 0.1 N, and 0.5 N and obtained identical readings. Stronger solutions than N caused precipitation. The entire amount of diluent is used at once, that is, 1 cc. of diluted packed red cells is discharged

into the volumetric flask containing about 50 cc. of 0.1 N HCl, then mixed, and the volume made up to the 100 cc. mark with 0.1 N HCl. The suspension is then allowed to stand 24 hours in the ice chest, thoroughly shaken again, for it must be remem-

TABLE III.
Hemoglobin.

Palmer's method.	Author's modification.
<i>per cent</i>	<i>per cent</i>
75	74
70	70
77	76
77	77
74	73
73	73
74	71
75	74
76	75
73	73
64	64
63	63
76	76
45	45
60	60
84	85
69	69

TABLE IV.

Van Slyke's oxygen capacity method.	Palmer's method.	Author's modification.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
113	113	114
117	118	117
99	98	99
124	124	124
109	108	109

bered that we are dealing with a suspension and not a solution, and then read. The method of hemoglobin determination is exactly like that of Palmer's method and the readings obtained are almost identical as illustrated by the figures given in Tables III and IV.

The source of light used for color comparison makes little difference. Light from a northern exposure or that originating from a nitrogen-filled bulb filtered through "Daylite" glass gives equally good readings. The slight turbidity of the acid hematin suspension, and it is very slight in a 1 per cent dilution, does not in the least interfere with an accurate color comparison.

Different standards have been prepared, 5, 10, and 20 per cent suspensions, defibrinated as well as whole blood, also blood from different species. In fact, standards have been made up from the same sample of blood, one diluted for carbon monoxide hemoglobin determinations and the other in the form of acid hematin suspensions. The standards have been checked up once a month as those for Palmer's method. Material for the 1 per cent dilution has been withdrawn from the stock bottles once each week, and the container resealed with paraffin. On prolonged standing some of the hematin settles to the bottom of the container but when the mixture is thoroughly shaken again the readings obtained are unchanged. It is of course very essential that all acid hematin suspensions, whether dilute or concentrated, are thoroughly mixed before using.

The keeping qualities, or rather stability of color density, are best demonstrated by Tables V, VI, VII, and VIII.

Standard IVa (Table V) faded 4 per cent during a time interval of 11 months. The change was apparent during the 1st month. In the same period of time the carbon monoxide standard had faded 16 per cent.

These tables (V, VI, VII, and VIII) demonstrate the keeping qualities of our acid hematin mixtures. Up to the present time the last three have remained practically unchanged. We shall continue to check up these suspensions once each month in order to determine just how long they will remain stable.

A 1 per cent standard diluted from Standard VIIa at the time of preparation, April 1, 1919, and simply kept in an Erlenmeyer flask in the ice chest still read 100 per cent on October 21, 1919. Another 1 per cent suspension diluted from the defibrinated sheep blood standard and kept under the same conditions as the one mentioned above also remained unchanged during the period of observation, 4 months. For exactly how long a period the 1 per cent dilution would remain stable we do not at present

know. We consider it safe to make up our 1 per cent suspensions from the stock mixture once each month. It may be of some importance that all these standards were preserved in an

TABLE V.

Standard IVa; Acid Hematin. Defibrinated Human Blood Prepared Nov. 25, 1918.

Date.	New standard prepared.	Standard IVa.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
<i>1918</i>			<i>per cent</i>	<i>per cent</i>	
Dec. 24.....	100	97	3	7	Carbon monoxide standard was prepared from the same sample of blood.
<i>1919</i>					
Jan. 24.....	100	97	3	11	
Mar. 5.....	100	96	4	11	
Apr. 1.....	100	97	3	16	
May 15.....	100	96	4	16	
June 23.....	100	97	3	16	
Aug. 6.....	100	96	4	15	
Sept. 7.....	100	96	4	15	
Oct. 21.....	100	96	4	16	

TABLE VI.

Standard Va; Acid Hematin. Defibrinated Sheep Blood Prepared Jan. 24, 1919.

Date.	New standard prepared.	Standard Va.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
<i>1919</i>			<i>per cent</i>	<i>per cent</i>	
Mar. 5.....	100	100	0	2	Carbon monoxide standard was prepared from the same sample of blood.
Apr. 1.....	100	100	0	9	
May 15.....	100	100	0	9	
June 23.....	100	100	0	8	
Aug. 6.....	100	100	0	10	
Sept. 7.....	100	100	0	10	
Oct. 21.....	100	100	0	10	

ice chest with fairly constant temperature, fluctuations rarely exceeding 1-4°C.

TABLE VII.

Standard VIa; Acid Hematin. Whole Dog's Blood Prepared Mar. 5, 1919.

Date.	New standard prepared.	Standard VIa.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
<i>1919</i>			<i>per cent</i>	<i>per cent</i>	
Apr. 1.....	100	100	0	7	Carbon monoxide standard prepared from the same sample of blood.
May 15.....	100	100	0	12	
June 23.....	100	99	1	13	
Aug. 6.....	100	100	0	15	
Sept. 7.....	100	100	0	14	
Oct. 21.....	100	100	0	Spoiled.	

TABLE VIII.

Standard VIIa; Acid Hematin. Defibrinated Dog's Blood Prepared Apr. 1, 1919.

Date.	New standard prepared.	Standard VIIa.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
<i>1919</i>			<i>per cent</i>	<i>per cent</i>	
May 15.....	100	100	0	0	Carbon monoxide standard prepared from the same sample of blood.
June 23.....	100	100	0	0	
Aug. 6.....	100	100	0	0	
Sept. 7.....	100	100	0	0	
Oct. 21.....	100	100	0	0	

III. Newcomer Method.—While this investigation was being carried on Newcomer published his new method of estimation of hemoglobin, details of which have been described above. A piece of this brown semaphore glass 0.96 mm. in thickness was tested and gave most satisfactory readings when compared with Palmer's figures as shown in Table IX.

A Duboscq colorimeter is used in this laboratory and the brown glass inserted above the plunger. The corresponding cup is partially filled with distilled water for the reasons mentioned by Newcomer. As a source of light for these hemoglobin determinations either the lamp containing "Daylite" glass or light from a northern exposure gave equally satisfactory results. While the colors of the glass 0.96 mm. in thickness and the acid hematin suspension of the blood to be tested matched satisfac-

torily, the color is very light, almost a lemon-yellow when matched. It is therefore quite evident that this is somewhat of a disadvantage for darker shades are more easily matched and of course there is no means of regulating the depth of standard color as one does when using liquid mixtures. For example, with our liquid standards set at 10 the resulting readings of the test fluids range usually between 10 and 13, while with the colored glass, at least with the piece of this particular thickness, the readings are around 5 to 7 and are not so accurate as those around 10 or 12 with the Dubosq instrument.

TABLE IX.

Palmer's method.	Newcomer's method (glass 0.96 mm. thick).	Difference.
<i>per cent</i>	<i>per cent</i>	
113	114	+1
116	114	-2
99	100	+1
107	108	+1
98	101	+3
117	118	+1
100	100	0
100	100	0
100	101	+1
100	103	+3
100	99.2	-0.8
Average difference		+0.73

The added advantage of course is that all artificial standards like this glass are supposedly permanent in color and this obviates the necessity of liquid standard preparations. Because of the pale color of the standard glass we purchased another piece somewhat thicker—1.02 mm.—hoping to obtain easier readings. While the color was slightly darker in this new piece our readings were not so accurate as is evident from the figures cited (Table X).

The difference in readings (Table X) was more than with the piece 0.96 mm. in thickness, an average of 0.73 higher with the former glass as compared to 2.4 points lower with the thicker one. We feel certain that this larger difference is due to the

fact that with the piece 1.02 mm. thick the color, although darker, is not so readily matched. The suspension of acid hematin demonstrates of course a very slight turbidity, the lack of which is very noticeable when using the heavier glass. The color of the latter is a clear yellowish brown while the acid hematin because of its slight opaqueness seems a somewhat different shade of brown. Some of the readings cited were made by different workers in the laboratory and the same difficulty was voiced by all that the colors do not seem to be quite the same. Two pieces of glass placed one on top of the other, each 1.02 mm. in thickness, increased the difference in readings considerably, as

TABLE X.

Palmer's method.	Newcomer's method (glass 1.02 mm. thick).	Difference.
<i>per cent</i>	<i>per cent</i>	
64	62	-2
63	60	-3
76	72	-4
45	47	+2
60	56	-4
84	81	-3
69	66	-3
Average difference		-2.4

well as the difficulty of exact color match. Newcomer (8) states that it is impossible to secure an artificial color match which runs true through a range of thicknesses. While the actual difference in thickness of the pieces of glass purchased—using of course only one at a time—seems slight, a decided difference in color match is apparent.

In view of the above mentioned difficulties we prefer using the liquid acid hematin standards.

DISCUSSION.

In summarizing our observations with the Palmer method it is evident that as long as the standard solutions are prepared once a month very accurate results may be obtained. The method itself is certainly simple and easily carried out by even

comparatively inexperienced laboratory workers. The main disadvantage lies in the color fading of the standard solutions. Among nine different standards observed for a period of from 2 to 13 months we have found but one solution which for nearly 7 months remained unchanged. The remaining eight all faded sufficiently to prohibit their use for accurate work, with one exception possibly—Standard VIII—which demonstrated a fading of only 5 per cent during a 5 months period. Considering the figures presented it seems that better results were obtainable with blood previously defibrinated than with whole blood. For a time interval of 3 months the standard solutions prepared from defibrinated blood demonstrated a fading of from 0 to 13 per cent as compared with 13 to 27 per cent evident in those mixtures originating from whole blood. We have never encountered any difficulty with reference to a true change of color, at least during a period of 6 months. During the last few months of observation a very slight brownish tinge was noticeable but never sufficient to interfere seriously with a color comparison, excepting perhaps Nos. I and VI, which were discarded after 8 months observation. The greatest change in color density in standards prepared from whole blood apparently takes place during the first 2 months and reaches its maximum during the 3rd month. From then on the change is but comparatively little and remains so, in some instances for a year or over. With the defibrinated blood, I think we have a standard of better keeping qualities; the change is not quite so pronounced. Although two of the defibrinated blood mixtures, Nos. III and IV, showed a fading of 11 per cent each during a time interval of 2 months, we have three others where the change was considerably less, one solution demonstrating but a 5 per cent loss of color within 5 months, another remaining unchanged for nearly 7 months, and a third fading 10 per cent in 9 months.

The type of hemoglobin evidently plays no important rôle, dog's blood apparently giving as satisfactory results as goat's, sheep's, or human blood; or rather the other species mentioned offer no more stable hemoglobin solutions than dog's blood.

Bacterial decomposition does not seem to be a very important factor, for blood obtained aseptically and the hemoglobin solution prepared with sterile precautions demonstrated no more

stable qualities than did those standards prepared with only the usual care and cleanliness.

It should be mentioned here that all these stated observations pertain to the stock solutions, the 20 per cent dilutions. We have never attempted to keep the 1 per cent dilutions prepared from the concentrated mixtures for more than a week, at least under experimental conditions existing here. A 1 per cent solution prepared will not fade within a week if kept on ice and resaturated with carbon monoxide each time the container is opened. After that time interval a change does take place which although not apparent to the eye is readily demonstrable when checked against the oxygen capacity method. Appleton (1) states that the 1 per cent solutions prepared by her began to deteriorate in from 2 to 4 weeks. During her investigation the diluted solution was kept saturated by a continual flow of gas. Why such a difference in stability occurs we do not know. Frequent opening of containers and resaturation with CO seem to give no better results than resaturation once a month. While a standard is being used for routine work it is necessary to open the container once each week in order to procure the necessary material for one 1 per cent dilution.

From the tables of acid hematin standards, it is readily seen that, while these acid hematin mixtures may change in time, they certainly have proved themselves to be much more stable than the carbon monoxide solutions. One may with perfect safety and with complete assurance of obtaining accurate results employ these acid hematin suspensions for 6 months at least. The stock mixtures above mentioned will of course be observed to determine just how long they actually remain unchanged.

The method of preparing blood for hemoglobin determinations in the form of acid hematin is slightly simpler than the carbon monoxide method, for the former makes unnecessary the extra step of saturation with carbon monoxide. The slight disadvantage is the time interval necessary for the maximum color of acid hematin to develop. In using large quantities of blood (at least much greater amounts than are used clinically) we have allowed 24 hours. This, however, is not necessary as a 1 hour interval gives accurate reading. We have observed no difference between figures obtained after 60 minutes standing and 24 hours.

The latter time happened to be more convenient in our experimental work. Newcomer published a table with his method showing the exact percentages of color development of acid hematin in given periods of time. He considers 40 minutes as safe.

Palmer in his publication states that blood of different species cannot be used for hemoglobin determinations in the form of acid hematin. As will be seen from our tables, we have standards prepared from human, dog's, and sheep's blood and have compared human blood with both dog's and sheep's blood standards without encountering any difficulty whatsoever. The comparisons have been made in all combinations possible with our material and readings have checked accurately. The figures presented in the tables readily answer the question of stability. While the latter may not be permanent or remain unchanged indefinitely, still it is much less time-consuming to prepare a fresh standard once every 6 months instead of once each month as we have had to do when employing the carbon monoxide method.

The Newcomer method would of course be the best solution of the entire problem, but as mentioned before the color match is not exact. The use of as simple a standard as a piece of colored glass certainly is a great advantage. The difference in color may not be so apparent to all eyes. The table accompanying the glass standard is an asset, as it definitely settles the question of time interval for development of the maximum color of acid hematin, and thus does away with one of the disadvantages of the earlier acid hematin methods.

Since the completion of this work a communication of Cohen and Smith (3) has appeared which confirms much of this work. They suggest the same standard solution because of its stability under army camp conditions.

CONCLUSIONS.

1. The Sahli hemoglobin method when using the color tubes accompanying the instrument gives very inaccurate results because of the decided variance in color density of the standard tubes, due to fading.

2. The Palmer method offers very satisfactory means of hemoglobin determinations if the standard solutions are freshly prepared. The method itself is very simple and may be successfully carried out by anyone familiar with colorimetry. The standard solutions prepared in the laboratory although carefully made have not been sufficiently stable to insure accurate determinations over periods of more than 3 to 4 weeks.

3. Newcomer's method obviates many difficulties heretofore observed with other procedures and gives good results with the glass 0.96 mm. in thickness, although the color is quite pale. When using the heavier glass, 1.02 mm. in thickness, the color match is only approximate and the figures obtained are not so satisfactory as those resulting from use of the thinner piece.

4. A method is presented applying Palmer's procedure to Sahli's principle which has proved most satisfactory. It removes the difficulty we encountered with Palmer's method, the lack of stability of color in the standard solutions. It has the advantage of an easier color match than that of red tint. The standards prepared have remained sufficiently unchanged for a period of 11 months to insure accurate hemoglobin determinations during this long period.

It may be suggested that for routine hospital work an acid hematin standard prepared in this way and kept at relatively constant temperature will remain unchanged for 8 months or longer. 1 per cent solutions may be prepared from time to time from the standard concentrated solution and this 1 per cent solution can be used to fill the standard tube of the common Sahli hemoglobinometer. This insures an accurate base line for hemoglobin determinations and with refilling of the Sahli tubes once a month will give accurate clinical determinations. Such clinical determinations are not the rule and are much to be desired.

BIBLIOGRAPHY.

1. Appleton, V. B., *J. Biol. Chem.*, 1918, xxxiv, 369.
2. Berezeller, L., *Biochem. Z.*, 1918, lxxxvii, 23.
3. Cohen, B., and Smith, A. H., *J. Biol. Chem.*, 1919, xxxix, 489.
4. Haessler, H., and Newcomer, H. S., *Arch. Int. Med.*, 1916, xvii, 806.
5. Haldane, J., *J. Physiol.*, 1899-1900, xxv, 295; 1900-1901, xxvi, 497.
Haldane, J., and Smith, J. L., *J. Physiol.*, 1899-1900, xxv, 331.

6. Hoppe-Seyler, F., *Z. physiol. Chem.*, 1891-92, xvi, 505.
Hoppe-Seyler, G., *Z. physiol. Chem.*, 1895-96, xxi, 461.
7. Lillendahl-Petersen, N., *Berl. klin. Woch.*, 1914, li, 551.
8. Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 465.
9. Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.
10. Sahli, H., *Lehrbuch der klinischen untersuchungs-methoden*, Leipsic, 4th edition, 1905, 655-664; 5th edition, 1909, 845; *Verhandl. Kong. innere Med.* 1902, xx, 230; *Jahresb. Fortsch. Thierchem.*, 1903, xxxii, 222.
11. Stäubli, C., *Münch. med. Woch.*, 1911, lviii, 2429.
12. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
13. Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1917-18, xlv, 573, 577.

BLOOD REGENERATION FOLLOWING SIMPLE ANEMIA

I. MIXED DIET REACTION

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This series of papers deals with the regeneration of red cells and hemoglobin following simple anemia and the influence of diet factors upon this reconstruction. It will be shown that the curve of hemoglobin regeneration can be influenced at will by various diet factors. We believe that it is desirable to mention at least two lines of investigation which are being followed in this laboratory. To determine the value of various food factors when given alone or combined with other substances. To determine further the few or many substances which promote speedy regeneration of hemoglobin and red cells or to ascertain the optimum food combinations which will give a maximum blood regeneration following simple anemia. Inorganic substances and certain drugs are being investigated and this work will be presented in its proper place. Some experiments will deal with splenectomized dogs as well as bile fistula dogs but we prefer to present at a later time the bulk of our work on splenectomized animals which deals with the relation of splenectomy to blood regeneration under fixed experimental conditions. A preliminary report covering a part of this anemia work has been published elsewhere (1).

This work on blood pigment regeneration forms an essential part in any study of "pigment metabolism of the body." It is obviously closely related to a study of *bile pigment excretion* which was first taken up in our work and has been reported in part in earlier publications (2), (3). It will be recalled that the excretion of bile pigments may be

influenced or modified by various diet factors. For example, meat will cause increased flow of bile but a decided drop in total bile pigments. Carbohydrate, on the contrary, will reduce the flow of bile but increase the total bile pigment output. Decreased functional activity of the liver is associated with a decided fall in bile pigment elimination. We have assumed on the basis of much experimental evidence that the liver plays a *constructive* rôle in the bile pigment output. We hope to show the same relationship on the part of the liver to the *constructive mechanism* of blood regeneration.

We wish to emphasize that a curve of blood pigment regeneration cannot be established without accurate determination of at least two factors,—hemoglobin and blood volume. With a knowledge of these two factors we can estimate the total volume of hemoglobin pigment in the body circulation—the “pigment volume.” Also with the hematocrit values we are able to compute the total volume of red cells in the body circulation. Reasonably accurate methods for the determination of circulatory blood volume are of recent development. A critical review of many factors in this blood volume work and comparison of various methods have been published from this laboratory very recently (4). The method used in this laboratory for accurate determinations of hemoglobin has been recently described by F. S. Robscheit (5).

Practically all these anemia blood regeneration experiments were performed upon dogs born and raised in our kennels—a bull dog cross which gives a very active, vigorous and healthy laboratory animal. Unless otherwise noted, the dogs were in fine normal condition during the entire experiment. These dogs will eat the food mixtures as a rule without any delay or wastage. They have been immunized against distemper and are kept in a separate room to obviate any cross infections by transient animals.

METHODS

The blood volume method used in these experiments has been described in detail elsewhere (4). In some of the earlier experiments dry oxalate was used for blood collection instead of isotonic fluid oxalate and in these experiments the calculated blood volume is too high. A note will be made in all such experiments as a correction cannot be introduced because the amount of solid oxalate and the corresponding shrinkage of cells was an unknown variable.

It may be stated in a word that the blood volume method consists in the introduction of a measured amount of a dye "brilliant vital red" into the blood stream. After a four minute period the dilution of the dye in the plasma is colorimetrically determined. "Brilliant vital red" has been furnished us through the courtesy of Dr. H. M. Evans of the Department of Anatomy and we wish to acknowledge many favors and valuable advice given us by Doctor Evans. The red cell hematocrit is read in an accurately calibrated centrifuge tube into which blood has been drawn, using an isotonic sodium oxalate solution. It is then a simple matter to calculate the plasma volume, red cell volume and total blood volume. This method can be quickly and accurately performed. It causes the dog a minimal degree of inconvenience, only that due to a hypodermic needle puncture of a vein, and the loss of only 35 cc. of whole blood. This is such a small amount of blood removed from the large circulating blood volume that we do not include it in our calculations and feel that no secondary anemia factors are added to complicate the reaction curve following the initial bleeding.

The hemoglobin determinations are made by means of a modification of Palmer's method, recently described in detail by one of us (5). This insures an accurate measure of the hemoglobin, as relatively large amounts of packed red cells are used. In some of the earlier experiments the Sahli hemoglobin tubes were used and in some instances these tubes had faded, giving hemoglobin values which were too high. The base line of any experiment although too high will not disturb the fairly accurate curve of regeneration which is more important. A footnote will be appended to all experiments in which the Sahli readings are given.

Red and white cell counts are made in the routine manner. Identical counts have been repeatedly obtained by venous puncture and from a freely bleeding ear puncture. The former is now our routine procedure.

The simple anemia is produced in the following manner: A simple blood volume is performed. The next day the dog is bled one-fourth the determined blood volume. This is easily done by inserting a needle into the jugular vein and aspirating the blood into a calibrated flask containing oxalate. The following day the same amount of blood is aspirated in exactly the same manner. During these two bleeding days and the next resting day the dogs are on a bread and milk diet. Following the resting day a second blood volume is done to determine the actual amount of anemia produced. The calculated and actual figures do not correspond but too many factors enter this equation to permit a

discussion at this time: for example, the reserve of blood cells thrown in from the marrow, to mention only one. At times a third bleeding is necessary if the reserve has been too great. A third blood volume is then done. The dog is then placed upon a fixed diet and complete blood volume, hemoglobin and blood cell determinations are done once each week thereafter. Special care is taken to insure a sufficient food ingestion based on the number of calories and nitrogen intake. The weight curve is a good index of the general nutrition.

The dogs are kept in individual cages which are comfortable and of suitable size to permit of much exercise. The cages are cleaned once daily by the attendant, but all feeding is done by the person in charge of the experiment. The mixed foods are usually eaten at once when placed each morning in the cage. Fluids are usually given by stomach tube. Water is furnished in the cage at all times except in metabolism experiments when it is usually given by stomach tube. It should be emphasized again that these dogs were raised in the laboratory and are therefore healthy, vigorous and very active at all times. The laboratory routine disturbs them not at all and the performance of the blood volume requires only a few minutes. They will eat all manner of food mixtures with relish and alacrity. Unless otherwise noted, these dogs are in their usual healthy, active condition throughout the entire experiment. "Mixed diet" in these experiments indicates a mixture of food materials obtained from the University Hospital, consisting of bones, bread, cooked meat, potato, rice, macaroni and general table scraps.

EXPERIMENTAL OBSERVATIONS

The dogs used in most of the experiments tabulated below are young animals in the active growth period,—one year of age or less. Such animals are increasing in size, weight and strength and the demand for tissue building or growth factors is acute. It is therefore of interest to keep this fact in mind during our analysis of the subsequent experiments. It might be assumed that the reconstruction of red cells in a rapidly growing animal might be handicapped by the tissue demand for normal growth factors. On the other hand, it may be argued that the growth capacity of the younger organisms might be greater as regards the construction of tissue cells, including the red cells. When we review the experiments to be submitted in subsequent communications it may be stated that the difference between the adult and the young dog is not great as regards the capacity of the animal to regener-

ate new red blood cells. This statement applies to young dogs between 6 and 12 months of age and takes into consideration the individual variations which are met with in different dogs. It is possible that this statement may not hold for pups less than 6 months of age. In general we may be safe in stating that if there is any difference between young and adult dogs, there is a slight difference in favor of the adult

TABLE 1

Blood regeneration—mixed diet. Dog 19-93. Bull pup, female, age 13 months

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIME'S BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
8/11	1780	1280	580	688	53.7	139	0.91	7,6	16,2	12.6	101	
8/12	Diet: Bread and milk											
8/12	Bled 320 cc. Slight distress. Injection 100 cc. N/1 salt solution											
8/13	Bled 320 cc. No distress											
8/15	614	986	732	249	25.3	62	0.97	3,2	12,8	13.0	76	Normal
8/15	Diet: Mixed diet											
8/21	658	1023	716	292	28.5	64	0.80	4,0	6,2	13.25	77	Diet poor in meat
8/28	962	1210	728	465	38.4	79	0.69	5,7	10,8	13.3	91	
9/4	1280	1308	724	572	43.7	98	0.82	6,0	10,0	14.1	93	More meat in food
9/11	1538	1420	673	733	51.6	108	0.64	8.4	8,8	14.3	99	
9/18	1612	1355	644	700	51.6	119	0.68	8,7	10,4	14.5	94	
9/25	1726	1444	681	748	51.8	120	0.69	8,7	12,6	15.5	93	
10/2	1830	1490	675	793	53.2	123	0.72	8,5	12,0	15.7	95	

dogs who at times seem to show a slightly shorter period of blood regeneration under similar circumstances.

The first three experiments (tables 1, 2 and 3) were performed upon three young dogs of the same litter, all of the same weight, activity and general appearance. It will be observed that there are individual differences even under these favorable conditions. It is possible that some of this variation may be explained by individual preferences of the

different dogs for various food factors. They were all given an excess of mixed food from which of course they could pick out the bits of food which they preferred. During much of this diet experiment the "mixed food" was poorer than usual in meat and bones. This explains the fact that the period of blood regeneration appears somewhat longer than in some of the subsequent experiments. The explanation for this fact is found in paper IV of this series, which shows the remarkable efficiency of a meat diet in promoting blood regeneration.

TABLE 1-B
Experimental history. Dog 19-93

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
				<i>kgm.</i>	
Begin 1/16/19	Rice, potato, milk	1435	126	10.95	Born July 11, 1918 Maximum regenera- tion 5 weeks
Bled 590 cc.		594	86	10.25	
End 3/7/19		1098	113	10.35	
Begin 3/17/19	Rice, potato, milk (re- peat)	1040	89	11.35	Table 42 Complete regenera- tion 4 weeks
Bled 502 cc.		566	82	11.15	
End 4/30/19		1313	107	11.15	
Begin 8/11/19	Mixed diet	1780	101	12.6	Table 1
Bled 640 cc.		614	76	13.0	
End 10/2/19		1830	95	15.7	

It is to be noted that all these three dogs increased markedly in body weight. It should be stated that this was a general growth with increase in size, length of limb and body, not merely a deposit of fat. One is not surprised to note a gradual increase in blood and plasma volume during the experiment. The total volume of red cells, hematocrit reading of red cells and hemoglobin follow curves which are parallel. The color index is almost constantly between 0.65 and 0.85 and there is not a very strong tendency in these experiments for the color index to drop much below normal in the first two weeks following the hemorrhage. We shall not attempt at this time to discuss the fluctuations in white blood cells which are tabulated.

The "pigment volume" is a convenient and expressive term which indicates the total volume of circulating or effective blood pigment in the blood stream at the time of estimation of blood volume and hemoglobin. The *pigment volume is the product of blood volume times per cent hemoglobin*. In all tables the *pigment volume* is the first value

TABLE 2

Blood regeneration—mixed diet. Dog 19-94. Bull pup, female, age 13 months

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
	cc.	cc.	cc.	per cent	per cent					kgm.	cc.	
8/11	1705	1242	572	65.8	53.0	137	0.78	8,8	7,6	11.0	113	
8/11	Diet: Bread and milk											
8/12	Bled 310 cc.											
8/13	Bled 310 cc. No distress											
8/15	540	1000	761	230	23.0	54	0.84	3,2	6,4	13.5	74	Normal
8/15	Diet: Mixed diet											
8/21	686	1090	768	306	28.1	63	0.67	4,7	8,6	13.65	80	Diet poor in meat
8/28	800	1194	812	370	31.0	67	0.78	4,3	6,0	13.4	89	More meat in diet
9/4	972	1292	843	443	34.3	75	0.66	5,7	12,0	13.9	93	
9/11	1032	1328	836	489	36.8	78	0.67	5,8	8,2	14.0	95	
9/18	1062	1205	736	465	38.5	88	0.69	6,4	6,2	13.8	87	More meat in diet
9/25	1226	1295	760	524	40.4	95	0.68	7,0	7,8	14.45	90	
10/2	1298	1276	710	554	43.4	102	0.74	6,9	17,4	14.55	88	
10/9	1520	1463	827	624	42.6	104	0.73	7,1	13,6	15.5	95	
10/23	1550	1463	823	620	42.4	106	0.74	7,2	8,8	16.25	90	

given as we believe it gives the best general index of the curve of blood regeneration.

Table 2, dog 19-94 is an experiment with a dog presenting some unknown abnormal factor. This dog at times shows an eosinophilia yet only an occasional parasite egg can be demonstrated in the feces.

Treatment by oil of chenopodium and santonin has yielded no results. The dog is not as well nourished as the others of this litter and at times presents a slight relative degree of anemia. This fact is to be considered in a study of the abnormally slow blood regeneration in this dog. In spite of this the dog gained about eleven pounds during the course of the experiment.

Table 3 at the start of the experiment shows the remarkably high figure (152 per cent hemoglobin) which may be observed in normal dogs. Red blood counts of 7 to 9 millions are the rule.

TABLE 2-B
Experimental history. Dog 19-94

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 1/16/19	Bread, 300	1250	126	<i>kgm.</i> 10.65	Born July 11, 1918
Bled 620 cc.	grams, milk	462	84	10.00	
End 3/7/19	500 cc.	899	94	11.70	Maximum regenera- tion 5 weeks
Begin 3/17/19	Bread, 300	1146	93	13.05	Table 27
Bled 608 cc.	grams, milk	542	78	12.40	
End 4/30/19	500 cc. (re- peat)	1031	99	13.20	Maximum regenera- tion 5 weeks
Begin 8/11/19	Mixed diet	1705	113	11.00	Table 2
Bled 620 cc.		540	74	13.5	
End 10/23/19		1550	90	16.25	

October 9. Two doses of oil of chenopodium, 48 hours apart. Few ova found. No worms expelled. Last dose followed by santonin mixture. No effect.

Table 4 shows an experiment upon a young dog (6 months) in which one week's diet of rice, potatoes and milk followed the bleeding—then the usual mixed diet. The dog did not eat the rice, potato and milk diet and lost much weight. Also she developed signs of mild distemper, but this soon cleared up after being put on a liberal mixed diet. There was then a rapid gain in weight as well as in blood regeneration. The leucocytosis is probably to be explained by the mild infection with distemper. The total regeneration of red cells is complete in one month.

TABLE 3

Blood regeneration—mixed diet. Dog 19-95. Bull pup, female, age 13 months

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
8/11	2088	1369	568	794	58.0	152	0.88	8,6	10,4	13.2	104	
8/11	Diet: Bread and milk											
8/12	Bled 342 cc.											
8/13	Bled 342 cc. No distress											
8/15	750	1005	700	300	29.8	74	1.03	3,6	8,4	13.65	74	Normal
8/15	Diet: Mixed diet											
8/21	843	1106	728	367	33.2	76	0.93	4,1	17,8	14.2	78	Diet poor in meat More meat in diet
8/28	829	1066	747	414	35.5	78	0.75	5,2	10,2	14.0	83	
9/4	954	1243	814	423	34.0	77	0.71	5,4	19,0	14.65	95	
9/11	1313	1338	726	600	44.8	98	0.65	7,5	13,2	14.5	92	
9/18	1325	1243	674	552	44.4	107	0.74	7,2	16,0	14.65	85	
9/25	1730	1412	678	720	51.0	122	0.74	8,2	13,0	15.25	93	
10/2	1874	1410	618	779	55.2	133	0.75	8,8	16,4	15.45	91	
10/9	1655	1343	654	676	50.3	123	0.66	9,0	14,4	15.70	86	

TABLE 3-B

Experimental history. Dog 19-95

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 1/16/19 Bled 564 cc. End 3/7/19	Rice, potato, milk	1232	100	11.35	Born July 11, 1918 Complete regeneration 5 weeks
		560	75	11.00	
		983	90	11.20	
Begin 3/17/19 Bled 566 cc. End 4/30/19	Rice, potato, milk (re- peat)	1092	88	12.80	Table 41 Complete regeneration 5 weeks
		570	75	12.45	
		1237	98	11.95	
Begin 8/11/19 Bled 684 cc. End 10/9/19	Mixed diet	2088	104	13.20	Table 3 Maximum regeneration 7 weeks
		750	74	13.65	
		1655	86	15.70	

TABLE 4

*Blood regeneration—mixed diet (following rice, potatoes and milk). Dog 19-96.
Bull pup, female, age 6 months*

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/16	1200	1025	483	540	52.3	117	0.58	10,1	20,8	10.15	101	
1/16	Diet: Crackermeal and milk											
1/17	Bled 256 cc.											
1/18	Bled 256 cc. No distress											
1/20	514	778	574	196	25.2	66	1.10	3,0	16,0	9.75	80	Normal
1/20	Diet: Boiled rice, 200 grams; potatoes, 200 grams, milk, 500 cc.											
1/27	422	826	604	218	26.4	51	0.48	5,3	12,4	8.60	96	Mild dis- temper
1/27	Changed to mixed diet*											
2/3	765	915	554	357	39.0	84	0.67	6,3	15,8	10.1	91	Recovered from dis- temper
2/12	1013	1080	574	500	46.3	94	0.65	7,2	12,0	11.35	95	
2/19	1243	1243	646	584	47.0	100	0.71	7,0	12,8	11.80	105	
2/28	1288	1108	537	554	50.0	116	0.73	8,0	9,2	11.95	93	

* Developed mild case of distemper. Refused to eat rice and potatoes.

TABLE 4-B

Experimental history. Dog 19-96

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 1/16/19	Rice, potato, milk for 1 week. Mixed diet for 4 weeks	1200	101	10.15	Born July 14, 1918
Bled 512 cc.		514	80	9.75	
End 2/28/19		1288	93	11.95	Table 4
Begin 3/17/19	Rice, potato, milk	1520	92	13.50	Table 44
Bled 620 cc.		527	72	12.40	
End 4/30/19		778	95	9.90	Maximum regen- eration weeks
Begin 8/11/19	Mixed diet	1750	98	13.65	Table 5
Bled 668 cc.		650	72	13.35	
End 10/2/19		2012	98	15.45	

Table 5 shows a second experiment on the same dog, 19-96, given in table 4. The first experiment was done at the age of 6 months and this experiment at the age of 13 months. The period of blood regeneration is longer in this experiment as we believe the correct explanation is the low meat content of the mixed diet during this period. This was also

TABLE 5

Blood regeneration—mixed diet. Dog 19-96. Bull pup, female, age 13 months

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
8/11	1750	1335	658	670	50.2	131	0.90	7,3	14,6	13.65	98	
8/11	Diet: Bread and milk											
8/12	Bled 334 cc.											
8/13	Bled 334 cc. No distress.											
8/15	650	964	684	275	28.5	67	0.90	3,7	21,6	13.35	72	Normal
8/15	Diet: Mixed diet											
8/21	660	993	686	297	29.9	66	0.80	4,1	12,0	13.9	71	Diet poor in meat More meat in diet*
8/28	801	1155	770	368	31.9	69	0.66	5,2	12,4	14.05	82	
9/4	1054	1340	852	474	35.4	79	0.72	5,5	16,2	14.7	91	
9/11	1255	1340	742	596	43.7	94	0.64	7,3	18,2	14.65	91	
9/18	1348	1225	639	575	46.9	110	0.65	8,5	10,8	14.85	83	
9/25	1511	1280	634	630	49.2	118	0.59	10,0	9,6	14.90	86	
10/2	2012	1517	665	846	55.7	133	0.68	9,8	14,0	15.45	98	

* Poikilocytosis of red cells of moderate degree. Experimental history, table 4-b.

noted in tables 1, 2 and 3, which experiments were all done at the same time. Our experiments are usually carried out in groups of four.

Table 6 illustrates several points. This dog had been under observation for some time under a variety of dietary conditions (see experimental history—table 6-b). A period of sugar feeding gave no blood regeneration (refer to table 16 in the following paper) and 2 weeks of meat feeding had caused a considerable rise in the pigment volume.

The increase in pigment volume brought this figure back to normal in 4 weeks of mixed diet. The blood volume figures are based on determinations made with dry oxalate. This procedure, as has been pointed out, causes a shrinkage of cells and a dilution of the dye which gives blood volume figures abnormally high. Note that the blood volume figures per kilo are 100 to 136 cc. This fact may account for some of the fluctuations in the estimated plasma volume.

TABLE 6

Blood regeneration—mixed diet (following metabolism experiment). Dog 17-28. Bull dog, female, adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/19	1620	1500	600	900	60	108	0.76	7,1	7,4	11.60	129	
	Diet: Sugar diet, January 19 to February 23. Lean meat diet, February 23 to March 10. Mixed diet, March 10 to May 7. (Refer to table 16)											
3/16	1010	1246	735	511	41	81	0.64	6,3	7,2	10.40	119	Good condition
3/23	973	1158	660	498	43	84	0.64	6,5	10,6	9.90	116	Good condition
4/13	1910	1645	757	888	54	116	0.71	8,2	22,0	12.30	134	
4/20	1385	1260	630	630	50	110	0.66	8,3	9,8	12.60	100	
5/7	1895	1709	769	940	55	111	0.66	8,4	13,2	12.50	136	Good condition

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

Table 7 is placed in this paper to give a comparison between the mixed diet experiments and fasting periods. It will be noted that during this fasting period the blood regeneration is very slight. This point will be discussed at length in the next paper.

DISCUSSION

From a review of the tables it is at once obvious that the *pigment volume* and total *red cell volume* after hemorrhage are below the expected values. There are several obscure factors in this reaction which call

TABLE 6-B

Experimental history. Dog 17-28

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 9/13/16	Bread and milk			kgm. 8.4	No blood volume data given
Bled 450 cc.				8.4	
End 10/25/16				8.8	Hb. and R.B.C. back to normal
Begin 1/19/17	Sugar and metabolism	1620	129	11.6	Table 16
Bled 750 cc.		549	87	10.4	Lean beef diet 2 weeks followed by mixed diet. Table 6
End 2/23/17		541	117	8.0	
Begin 5/7/17	Sugar + R.B.C., metabolism	1895	136	12.5	Table 76
Bled 854 cc.		594	76	12.0	
End 6/18/17		1085	154	8.3	Put on bread and milk diet
Begin 9/11/17	Sugar 2 weeks Sugar 2 weeks + diamino-acid of gelatin	1650	113	14.0	
Bled 794 cc.		751	84	13.5	
End 10/19/17		1040	106	10.0	Beef heart 3 weeks, mixed diet 3 weeks
Begin 6/3/18	Desiccated kidney, bread and milk	1739	73	17.65	
Bled 949 cc.		792	62	16.50	(3 bleedings)
End 6/28/18		1300	74	16.10	
Begin 8/9/18	Sugar and Hb. intraperitoneally	2417	93	17.00	Table 80
Bled 1076 cc.		756	66	15.90	(3 bleedings)
End 11/13/18		2000	106	15.05	
Begin 12/2/18	Lean meat and gelatin	2118	105	15.70	Table 51
Bled 1140 cc.		708	82	15.05	(3 bleedings)
End 1/22/19		1380	99	14.65	
Begin 5/1/19	Fasting	2018	97	15.90	Table 14
Bled 980 cc.		763	71	14.75	(3 bleedings)
End 5/21/19		1007	86	11.50	Found dead. Renal calculi

for discussion. Let us take a type experiment with simple values. A dog with a hemoglobin of 100 per cent, hematocrit of 50 per cent and blood volume of 600 cc. will have a pigment volume of 600. On two successive days the dog is bled one-fourth of the estimated blood volume. Allowing for replacement of plasma which is known to take place, we should expect after these bleedings a hemoglobin of 55 per

TABLE 7

Blood regeneration—fasting. Dog 17-27. White bull mongrel, female, adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
3/11	2100	1500	735	766	51	140	0.99	7,1	10,4	15.8	95	
3/13	1627	1122	561	561	50	145	1.03	7,0	8,0	15.7	72	*
3/13	Diet: Bread and milk											
3/13	Bled 375 cc. No distress											
3/14	Bled 375 cc. No distress											
3/16	1005	1116	770	346	31	90	1.05	4,3	12,4	15.5	72	*
3/16	Fasting begun											
3/22	1165	1153	726	427	37	101	0.90	5,6	6,8	13.8	76	*
3/29	1086	1075	656	419	39	101	0.67	7,5	6,2	12.3	88	*
4/4	1213	1054	622	433	41	115	0.65	8,8	6,8	11.6	91	*
4/10	1240	1000	620	380	38	124	0.95	6,7	6,2	10.7	93	Excellent condition

* Slight anisocytosis.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes. Experimental history, table 15-b. See below.

cent and total volume of red cells 165 cc. The plasma is constant under such conditions and will be as normal, 300 cc. volume. The blood volume therefore is 465 cc. and pigment volume 256 (465×0.55) which is 43 per cent of the pigment volume at the beginning of the experiment. If there is no reserve red cell influx we should expect a red cell volume of 55 per cent and a pigment volume of 43 per cent normal following two bleedings of one-fourth the total blood volume. The tables

show figures which are consistently below the expected values. This calls for a broad discussion of the various methods of blood volume determination and possible errors inherent in the various methods. We do not wish to review all these points at this time but hope soon to do so in connection with experiments dealing with the actual technique of blood volume determination.

Two possibilities are to be mentioned, however, in this discussion. First the blood volume values may be too high, calculated by the dye method, and the actual bleedings represent more than 50 per cent of the total blood volume. Second, the possibility that the body has the power to modify the ratio of cells to plasma in different parts of the body under normal or abnormal conditions. It is easy to mention these two possibilities but very difficult to adduce experiments which are conclusive. For example in the abnormal condition of shock it seems clear that there is a remarkable disturbance in the ratio of cells to plasma in different parts of the body.

It may not be wise to pursue this question further but best merely to call the reader's attention to the fact that there is a discrepancy between the expected and actual values for red cell volume and pigment volume after unit hemorrhages. This point will be taken up again. The *pigment volume* figures, however, give a clear-cut index of the curve of blood regeneration.

SUMMARY

The term *pigment volume* used in these papers is equivalent to blood volume times per cent hemoglobin. This means the volume of available red cell pigment circulating in the body at the time of blood volume determination.

Given a uniform degree of anemia we may observe the curve of red blood cell regeneration as influenced by a variety of diet factors. Anemia is produced by bleeding the dog one-fourth of the determined blood volume on each of two successive days.

Under these experimental conditions a diet of mixed table scraps will effect complete blood regeneration to normal in a period of 4 to 7 weeks.

Under similar experimental conditions there will be little blood regeneration during a fasting period—mainly a maintenance factor equivalent to the normal daily wastage of red cells.

BIBLIOGRAPHY

- (1) HOOPER AND WHIPPLE: This Journal, 1918, xlv, 573, 576.
- (2) HOOPER AND WHIPPLE: This Journal, 1916, xl, 332.
- (3) WHIPPLE AND HOOPER: This Journal, 1917, xliii, 258.
- (4) HOOPER, SMITH, BELT AND WHIPPLE: This Journal, 1920, li, 205.
- (5) ROBSCHUIT: Journ. Biol. Chem., 1920, xli, 209.

BLOOD REGENERATION FOLLOWING SIMPLE ANEMIA

II. FASTING COMPARED WITH SUGAR FEEDING

Analysis of "Sparing Action of Carbohydrates"

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That there is a distinct difference between the blood regeneration during *fasting periods* as compared with *sugar diet periods* comes out from an analysis of the experiments given below (table 25-a). This difference in blood regeneration is not great but is distinctly in favor of the fasting condition,—in other words, *a dog will form more red cells and hemoglobin during a fasting period than during a similar period of sugar feeding*. In neither case is any nitrogenous material taken into the body, so whatever hemoglobin and red cell stroma may be formed must be constructed in the body from body protein or protein split products. The well known "sparing action of carbohydrates" must be considered in the analysis of these experiments given below. It appears that these experiments can be explained most satisfactorily on the basis of a certain protection or sparing of body protein on the part of the sugar, associated with a definite amount of conservation of protein split products.

Having established the curve of blood regeneration which is the result of a mixed diet subsequent to the anemia period, we wish to present experiments to show the type reaction associated with fasting or sugar feeding. In making any analysis of results it is necessary to know how much reserve capacity the normal dog possesses—how much regeneration of red cells or hemoglobin can be effected during periods of fasting or sugar feeding. We must take into consideration too the daily wear and tear of the red cells which is not an accurately established factor. We do not know the life history of the red cell in the normal or anemic dog. We do know the duration of life of the red cell in the normal human being (1), but this life cycle may be different in disease. The

dog's red cells are extremely fragile and this may or may not indicate a shorter life cycle for dog than the established 30-day period for normal human beings. Data on this point are very much to be desired but in their absence we must postulate an unknown factor of red cell or hemoglobin replacement which is present in all our tables.

Any diet, therefore, which is capable of giving a rising curve of blood regeneration following simple anemia is doing two things. The diet is responsible for a replacement of the red cells (3 to 5 per cent per day) which are worn out day by day, as well as the rise in general level of red cell volume above the anemia level. For the sake of analysis we may assume that the replacement value for human beings and dogs is the same—about 3 per cent per day or complete replacement of the total volume of red cells in approximately 30 days. Under most favorable conditions we may see the volume of red cells regenerate from an anemia level of one-third or one-half normal back to 100 per cent within 4 or 5 weeks. To supply a deficit of 50 to 65 per cent of its red cell volume the body requires 30 days or more over and above its maintenance of red cell wastage. This wastage (wear and tear of red cells) in human beings may be 100 per cent in 30 days. This indicates the importance of this replacement factor and further emphasizes the fact that our curves show the reaction of the body *in excess* of this wastage or replacement value.

When we note a falling curve of hemoglobin and red cells after a long diet period we need not hastily postulate hemolysis or blood destruction from some hypothetical toxin. It may be safer to consider the possibility that the body can form no more hemoglobin or red cells to repair the daily wastage. Even the replacement fraction is not being supplied and the curve subsides gradually depending upon the life cycle of the red cells remaining.

EXPERIMENTAL OBSERVATIONS

The experimental procedures have been described in detail in the preceding communication. The majority of these dogs have been under observation in the laboratory since birth and we have studied their reaction to simple anemia following hemorrhage as influenced by a variety of diets. Some of these observations precede and others follow these tabulated fasting or sugar feeding experiments. The experimental histories given with each animal give a review of the many experiments done on the same dog. The value of comparison under these conditions

is greater than obtains in a series of isolated experiments. The dogs were all very fat and well nourished at the beginning of these experiments and were able to tolerate the fasting or sugar periods without disturbance of health or activity.

During the metabolism experiments the dogs were kept in standard metabolism cages constructed with sharp pitch of the cage bottom to insure rapid and complete drainage of urine. The dogs were catheterized every 24 hours and the catheterized specimen, bladder washings

TABLE 8

Blood regeneration—fasting—metabolism—splenectomy. Dog 17-37. White bull mongrel, female, age 10 months

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
3/26	927	850	459	391	46	109	0.80	6,8	9,4	10.60	80
3/26	Diet: Bread and milk										
3/28	Bled 212 cc.										
3/29	Bled 212 cc.										
3/30	313	626	451	175	28	50	0.68	3,7	15,4	10.40	60
3/30	Fasting begun										
4/2	403	651	475	176	27	62	0.74	4,2	6,4	9.50	70
4/9	492	769	500	269	35	64	0.64	5,0	7,2	8.50	90
4/16	532	729	474	255	35	73	0.66	5,5	5,0	7.70	96
4/23	540	772	502	270	35	70	0.66	5,3	7,8	6.90	112

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

and cage urine were made up to a uniform volume. A mixed specimen was preserved and duplicate Kjeldahl analyses made. A few cubic centimeters of acetic acid in the cage collection bottle insured an acid reaction in the urine. With care a male or female dog may be catheterized daily without causing any cystitis. After the catheterization the water or sugar solution was given by stomach tube.

Fasting experiments. The two preceding experiments (tables 8 and 9) are to be compared as the experiments were done at the same time.

TABLE 8-A
Total urinary nitrogen—fasting. Dog 17-37

DATE, 1917	TOTAL NITROGEN 24 HOURS URINE	URINE TOTAL 24 HOURS	WEIGHT	REMARKS
	<i>grams</i>	<i>cc.</i>	<i>pounds</i>	
March 31	2.80	650	21.5	0 feces
April 1	2.30	405	21.1	Trace of feces
2	2.58	433	20.8	0 feces
3	3.19	345	20.6	0 feces
4	2.97	395	20.3	0 feces
5	3.56	418	19.9	Slight diarrhea
6	2.83	460	19.6	0 feces
7	2.88		19.3	0 feces
8	2.49	427	19.0	0 feces
9	2.80	385	18.8	0 feces
10	2.91	473	18.3	0 feces
11	2.69	359	18.3	0 feces
12	2.91	423	17.9	Trace of feces
13	2.60	371	17.6	0 feces
14	2.60	429	17.5	0 feces
15	2.85	411	17.3	0 feces
16	2.86	414	17.1	0 feces
17	2.60	447	16.8	0 feces
18	2.77	458	16.5	0 feces
19	3.19	396	16.3	0 feces
20	3.02	421	16.1	Trace of feces
21	3.42	416	15.8	0 feces
22	3.56	445	15.7	0 feces
23	3.86	471	15.1	0 feces

Dog given 400 cc. of water daily by stomach tube.

TABLE 8-B
Experimental history—dog 17-37—splenectomy

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 12/15/16	Sugar + gela- tin	630	66	<i>kgm.</i> 10.5	Maximum regenera- tion 2 weeks, then lean meat diet 3 weeks. Mixed diet
Bled 350 cc.				10.2	
End 1/11/17		544	84	8.1	
Begin 3/26/17	Fasting metab- olism	927	80	10.6	Table 8
Bled 424 cc.		313	60	10.4	Table 73
End 4/23/17		540	112	6.9	Bread, milk, Bland's pills, 11 weeks. Slight regenera- tion

Splenectomy 10/23/16.

The dogs are of the same litter but one had been splenectomized. We can make out no difference in the reaction of the splenectomized dog as compared with the control under these experimental conditions. During the fasting period there is a steady rise in hemoglobin, red cell hematocrit, red cell count, total red cell volume and pigment volume. The rise is most pronounced during the first week, as a rule. It is noted that the plasma volume remains constant or decreases slightly. This

TABLE 9

Blood regeneration—fasting—metabolism. Dog 17-38. White bull mongrel, female, age 10 months

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
3/26	1395	1090	490	600	55	128	0.80	8,0	15,8	9.8	111
3/26	Diet: Bread and milk										
3/28	Bled 272 cc.										
3/29	Bled 272 cc.										
3/30	443	738	531	207	28	60	0.73	4,1	12,2	9.3	79
3/30	Fasting begun										
4/2	532	819	573	246	30	65	0.74	4,4	10,6	8.6	95
4/9	491	723	441	282	39	68	0.65	5,2	9,6	7.5	96
4/16	585	770	439	331	43	76	0.62	6,1	12,0	6.8	113
4/20	585	750	420	330	44	78	0.51	7,6	11,4	6.3	119

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

Experimental history, see table 20-b.

fact accounts for the increase in blood per kilo, due to loss of body weight.

The nitrogen figures for total urine are of interest. The daily output is fairly constant until we reach the last 4 days of the experiment which show a distinct increase above normal. It is possible that this increase represents the body protein disintegration which may be observed after a long period of fasting, giving rise to the premortal rise in nitrogen. Both these dogs recovered the lost weight promptly when placed upon

a bread and milk diet. There is no evidence for an increase in body protein katabolism to supply the essentials for hemoglobin construction. The same remarks apply to the following experiment (table 10).

The above experiment (table 10) shows a considerable increase in red cells from 5,200,000 to 7,000,000 with an unchanged red cell hematocrit. Poikilocytosis is marked during this period and there is evidence

TABLE 9-A
Total urinary nitrogen—fasting. Dog 17-38

DATE, 1917	TOTAL NITROGEN 24 HOURS URINE	URINE TOTAL 24 HOURS	WEIGHT	REMARKS
	<i>grams</i>	<i>cc.</i>	<i>pounds</i>	
March 31	3.42	720	19.4	0 feces
April 1	2.16	412	19.1	0 feces
2	2.18	471	18.8	Trace of feces. Vomited
3	2.77	236	18.6	Moderate feces
4	2.74		18.1	0 feces
5	2.94	430	17.9	0 feces
6	2.86	442	17.7	0 feces
7	3.25	428	17.3	Slight diarrhea
8	2.86	422	17.1	0 feces
9	2.66	432	16.7	0 feces
10	2.97	403	16.3	Trace of feces
11	2.46	413	16.2	0 feces
12	2.83	434	15.9	Slight diarrhea
13	2.72	383	15.7	0 feces
14	2.72	443	15.5	0 feces
15	3.05	446	15.1	0 feces
16	3.58	418	14.9	0 feces
17	3.42	408	14.7	0 feces
18	3.25	441	14.4	0 feces
19	4.14	441	14.1	0 feces
20	4.09	726	13.8	0 feces
21	4.17	517	14.3	Slight diarrhea

Dog given 400 cc. water daily by stomach tube.

for a certain amount of fragmentation of the red cells in this and other experiments of similar nature. In some instances there is evidence for a faulty construction of red cells during periods of stress when red cell regeneration is being accomplished with difficulty on a limited diet. It will be possible with the accumulation of much data to ascertain which diets favor stroma construction and which diet factors accelerate hemoglobin construction. It is evident at present that these two fac-

tors do not always run parallel under experimental conditions, as well as in disease.

TABLE 10

Blood regeneration—fasting—metabolism. Dog 16-160. Bull mongrel, female, age 2 years

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cen	per cent				kgm.	cc.	
5/3		1241	633	590	47.6					10.45	119	
5/3	Diet: Bread and milk											
5/6					45.2	90				10.00		
5/20	1316	1125	523	591	52.5	117	0.70	8,3	14,2	10.60	106	R.B.C. frag- ment++
5/21	Bled 281 cc.											
5/22	Bled 281 cc.											
5/24	482	752	492	250	33.2	64	0.55	5,8	14,8	10.20	74	
5/25	Fasting begun. Metabolism											
5/31	585	688	455	226	32.8	85	0.82	5,2	6,0	9.10	76	* Poik.
6/5	591	672	437	225	33.5	88	0.72	6,1	6,8	8.40	80	* Poik.++
6/12	636	707	457	243	34.3	90	0.64	7,0	8,2	7.55	94	* Poik.++
6/12	Diet: 200 grams bread and 300 cc. milk. Metabolism discontinued											
6/18	696	791	510	274	34.6	88	0.88	5,0	5,4	8.15	97	* Poik.++
6/26	451	550	360	184	33.5	82	0.68	6,0	9,0	8.20	67	* Slight poik.
6/26	Diet: Changed to mixed diet											

* Poikilocytosis of red cells.

Experimental history, see table 18-b.

This second group of experiments (tables 11, 12 and 13) presents several factors in common. During the fasting period the blood regeneration was notable in two experiments during the first week following the bleeding. In fact this level was scarcely increased during the sub-

sequent fasting period. This rapid increase during the first week may be explained by some reserve factor which is called in during the emergency period. The following weeks show little increase in pigment volume because the body can only supply the material needed to replace the daily wear and tear on the red cells. The third experiment (table 12) shows a more gradual rise in the pigment volume, hematocrit and hemoglobin during the entire fasting period.

TABLE 10-A

Total urinary nitrogen—fasting. Dog 16-160

DATE, 1918	TOTAL NITROGEN 24 HOURS URINE	URINE TOTAL 24 HOURS	WEIGHT	REMARKS
	<i>grams</i>	<i>cc.</i>	<i>pounds</i>	
May 26	1.76	270	21.8	Some water vomited
27	1.90	120	21.4	About 50 cc. vomited; 0 feces
28	1.85	145	20.8	No vomiting. 0 feces; dog very active
29	2.07	113	20.6	No vomiting. 0 feces
30	2.02	155	20.3	0 feces
31	2.10	188	20.1	0 feces
June 1	2.02	199	19.8	0 feces
2	1.96	134	19.3	0 feces. 300 cc. water
3	2.44	167	19.1	0 feces. Good condition
4	2.16	206	18.9	0 feces
5	2.41	180	18.5	Solid feces
6	2.13	207	18.3	Little feces
7	2.02	221	17.9	0 feces
8	1.79	191	17.6	0 feces
9	1.90	186	17.4	0 feces
10	2.02	181	17.3	0 feces
11	1.90	178	17.0	0 feces
12	2.30	190	16.6	Feces + in urine. Dog in excellent condition
13	1.73	225	16.4	Soft feces

Dog given 200 cc. water daily by stomach tube.

All three experiments show a rapidly developing "dietary deficiency disease" which develops after a period of bread and milk feeding subsequent to the fasting period. This dietary deficiency condition is characterized by ulcerated mucous membranes and much gastro-intestinal disturbance. We are inclined to the opinion that this condition is analogous to scurvy in human beings. In the near future we hope

to report a series of experiments bearing upon this point, making clear the factors concerned in the development of this abnormal condition as well as its cure and prevention in the dog.

TABLE 11

Blood regeneration—fasting. Dog 18-103. Brindle bull, female, age 1 year

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
8/28	1540	1115	552	547	49.0	138	0.97	7,1	16,0	15.45	72	
8/28	Diet: Crackermeal and milk											
8/28	Bled 279 cc.											
8/29	Bled 279 cc.											
8/31	810	965	650	311	32.2	85				14.95	65	
8/31	Bled 241 cc.											
9/3	590	955	708	242	25.3	62	0.94	3,3	26,0	14.35	65	* Poik.
9/3	Fasting begun											
9/10	946	1076	692	358	33.3	88	0.72	6,1	13,0	13.15	82	Good con- dition
9/16	852	882	552	321	36.4	97	0.88	5,5	11,4	12.15	73	
9/25	745	774	484	275	35.5	96	0.69	7,0	7,2	10.90	71	
9/30	700	784	485	295	37.7	89	0.57	7,8	13,2	10.30	76	
9/31	Diet: 250 grams crackermeal, 300 cc. milk											
10/11	699	889	572	311	35.0	79				11.30	79	* Poik. +
10/18	692	822	544	274	33.3	84	0.74	5,7	4,8	10.25	76	
10/18	Diet: Mixed diet											
10/22	Death from dietary deficiency disease											

* Poikilocytosis of red cells.

No previous anemia experiments on this dog.

TABLE 12

Blood regeneration—fasting (followed by rice, bread and milk, then yeast). Dog 18-114. White bull, female, adult

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
5/1	2550	1830	698	1115	60.9	139	0.75	9,3	12,6	13.95	131	
5/1	Diet: Bread and milk											
5/2	Bled 458 cc.											
5/3	Bled 458 cc. No distress											
5/5	620	1000	690	301	30.1	62	0.86	3,6	14,0	12.60	79	
5/6	Fasting begun											
5/14	890	1043	619	394	37.8	85	0.76	5,6	8,0	11.30	92	* Poik.
5/21	899	968	598	375	38.7	93	0.86	5,4	6,0	10.45	93	* Poik.
5/28	1103	1077	588	478	44.4	102	0.69	7,4	6,4	9.55	108	
5/28	Diet: 200 grams bread, 300 grams rice, 500 cc. milk											
6/4	1166	1166	654	502	43.1	100	0.62	8,1	6,6	10.45	115	* Poik.++
6/5	Diet: 30 grams compressed yeast, 100 grams bread, 500 cc. milk											
6/11	1070	1084	609	464	42.8	99	0.56	8,9	7,4	10.35	105	* Poik++
6/18	1232	1109	572	526	47.4	111	0.57	9,8	6,0	10.30	107	* Poik.†
6/25	1167	1094	547	537	49.1	107	0.54	10,5	10,4	10.25	107	
7/1	1148	1125	594	516	45.9	102	0.65	7,8	11,6	10.00	112†	
7/1	Diet: Changed to mixed diet. Extra food. Recovery—4 days											

* Poikilocytosis of red cells.

† June 16: Mucous colitis. No yeast given; milk boiled.

June 17: No mucus; 10 grams yeast given; milk boiled.

June 18: No mucus; 20 grams yeast given; 300 cc. fresh milk.

‡ Beginning ulceration of mucous membrane of mouth. Beginning salivation.

Autopsy (see table 14)

Dog 17-28. White bull, female. See experimental history, table 6-b.

May 22, 1919. Found dead and cold. Blood clotted. Left ventricle slightly hypertrophied. Lungs and pleurae negative. Slight hypostatic congestion in right lower lobe. Spleen soft and flabby; normal size; pale pink-gray and cellular. Peritoneal cavity is normal. No pigmentation and no adhesions. Gastro-intestinal tract not opened, but superficially not abnormal. Liver normal size and color; indefinite hazy patches in which lobules look washed out (0.5 to

TABLE 12-B
Experimental history. Dog 18-114

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 4/24/18 Bled 279 cc. End 5/29/18	Cooked liver, bread, and milk	553 435 791	94 105 84	7.85 7.65 9.60	Slight anemia Table 63 Maximum regeneration 2 weeks. Mixed diet
Begin 8/14/18 Bled 816 cc. End 9/27/18	Powdered liver, cracker-meal and milk	1773 770 2190	106 95 112	12.20 11.75 12.90	(3 bleedings)
Begin 11/14/18 Bled 976 cc. End 12/11/18	Cooked liver only	1640 592 1902	105 81 111	13.50 12.50 13.65	Table 61 (3 bleedings) Complete regeneration 3 weeks
Begin 5/1/19 Bled 916 cc. End 5/28/19	Fasting	2550 620 1103	131 79 108	13.95 12.60 9.55	Table 12

2 cm. in diameter). Bladder contains a little syrupy pus-like urine; slight cystitis. Left kidney large and hard; few cysts on surface; large stone fills pelvis (2 x 10 cm. \pm). Right kidney shrunken to small size (2 x 3 x 5 cm.); stone in pelvis. Marrow of femur almost all fat. Marrow of rib is normal. Pancreas is small and warty looking—chronic change involves all of lower arm and head, all but distal fourth of upper arm. No evidence of acute process. Ovaries negative. Uterus shows sites of placental attachment. Urine: obtained from bladder at post-mortem. Small amount, mostly pus. No sugar. Few hyaline casts. Many pus cells and small round epithelial cells, few partially destroyed red cells. Bacteria abundant.

The experiment given in table 14 is complicated by nephritis, pyelitis and bilateral renal calculi. The blood regeneration during the two weeks preceding death was normal in every way. If anything, the

TABLE 13

Blood regeneration—fasting (followed by rice and bread and yeast vitamine). Dog 18-116. White bull, female, adult

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
5/1	2620	1930	852	1050	54.4	133	0.85	7,8	8,0	18.75	103	
5/1	Diet: Bread and milk											
5/2	Bled 483 cc.											
5/3	Bled 483 cc. No distress											
5/5	778	1245	884	355	28.5	62	0.79	3,9	12,8	17.45	71	
5/6	Fasting begun											
5/14	1178	1357	818	525	38.7	87	0.84	5,2	14,0	15.95	85	* Poik.
5/21	1025	1205	742	434	36.0	85	0.75	5,7	9,4	14.85	81	* Poik.++
5/28	1176	1233	720	519	40.7	95	0.73	6,5	7,6	13.85	89	* Poik.++
5/28	Diet: 300 grams rice, 200 grams bread											
6/4	1109	1265	798	462	36.5	88	0.62	7,1	9,8	14.75	86	* Poik.++
6/5	Diet: 1 gram yeast vitamine,† 100 grams bread, 500 cc. milk											
6/11	1065	1232	761	458	37.2	86	0.70	6,1	8,2	14.65	84	* Poik.+
6/18	1368	1323	730	580	43.8	103	0.54	9,6	10,2	14.60	91	* Poik.+
6/25	1280	1380	780	594	43.0	93	0.51	9,2	5,6	14.05	98	* Poik.++
6/25	Diet: Changed to mixed diet. Developed dietary deficiency disease											
6/30	Death											

* Poikilocytosis of red cells.

† Vitamine prepared according to Seidell's method (2).

blood pigment production was above the average. This is of some interest because of the frequent occurrence of anemia in human beings associated with advanced chronic nephritis. This dog's death was

certainly due to renal injury and insufficiency, but the picture was not that of a primary essential chronic nephritis. In this single instance the development of a subacute renal disease did not impair the function of the organs of the body which are responsible for blood and hemoglobin regeneration.

Sugar feeding experiments. The first three experiments are similar and give strong evidence to show that there is very little regeneration of red cells and hemoglobin during a sugar feeding period. The reac-

TABLE 13-B
Experimental history. Dog 18-116

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 4/24/18	Meat extract, bread and milk	903	99	<i>kgm.</i> 9.75	Table 65
Bled 540 cc.		422	89	9.65	(3 bleedings)
End 5/29/18		865	89	10.85	Mixed diet 2 weeks. Complete regener- ation
Begin 8/14/18	Thyroid, crackermeal and milk	1842	95	14.20	
Bled 944 cc.		1055	98	14.25	(3 bleedings)
End 10/24/18		1640	114	11.85	Complete regenera- tion 7 weeks
Begin 12/2/18	Beef heart and liver	2120	115	14.90	Table 52
Bled 1160 cc.		776	90	13.95	(3 bleedings)
End 12/30/18		2270	106	15.50	
Begin 5/1/19	Fasting	2620	103	18.75	Table 13
Bled 966 cc.		778	71	17.45	
End 5-28-19		1176	89	13.85	

tion is constantly in favor of the fasting period during which time the dog can make a definite gain in red cells and hemoglobin, in excess of the maintenance requirements. In the first two experiments (tables 15 and 16) we have control observations in the same dogs during fasting periods (tables 7 and 14). The regeneration is distinctly more during fasting. In all three experiments the gain in pigment volume is present in the first week and we may wish to assume some emergency reserve to account for this reaction. The following weeks show little

or no subsequent gain and may even show a falling off which indicates that the body cannot fabricate sufficient hemoglobin and red cells for its daily needs.

Splenectomy (table 17) done some time before this experiment does not appear to modify the reaction of the red cells and hemoglobin under the conditions of these experiments.

TABLE 14

Blood regeneration—fasting—nephritis and renal calculi. Dog 17-28. White bull, female, adult

DATE 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
5/1	2018	1550	706	829	53.5	130	0.76	8,6	7,0	15.9	97	
5/2	Diet: Bread and milk											
5/2	Bled 390 cc.											
5/3	Bled 390 cc. No distress											
5/5	763	1040	686	349	33.5	73	1.14	3,2	9,4	14.75	71	
5/6	Bled 200 cc. No distress											
5/8	631	956	648	295	30.8	66	0.94	3,5	8,2	14.25	67	
5/8	Fasting begun											
5/14	903	1041	643	415	39.9	87	0.79	5,5	7,8	13.0	80	*
5/21	1007	988	535	432	43.8	102	0.74	6,9	8,2	11.5	86	
5/22	Found dead. Autopsy given below											

* Dog is sick, weak and thirsty; distended abdomen.

Experimental history, see table 6-b.

The three metabolism tables (tables 15-a, 16-a and 17-a) in general show the characteristic reaction in urinary nitrogen. In every instance at the beginning when the anemia reaction is most intense we note the expected drop in urinary nitrogen when sugar is administered. The "sparing action of carbohydrate" is not disturbed by the presence of this degree of secondary anemia. The first and second experiments

show a distinct rise in urinary nitrogen associated with the bleeding periods. These dogs showed considerable distress during the second bleeding period and it is highly probable that more than one-fourth of the total blood volume was removed on these two days (note the high figure, 130 cc. per kgm., for blood volume as determined by the dry oxalate method). We are inclined to attribute this rise in urinary nitrogen to the shock of the bleeding—to the tissue injury produced by

TABLE 15

Blood regeneration—sugar feeding—metabolism. Dog 17-27. White bull mongrel, female, adult

DATE 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/19	1630	1507	603	904	60.0	108	0.71	7,6	9,0	11.60	130	Fasting
1/20	Bled 375 cc.											
1/22	Bled 375 cc.											
1/24	598	950	627	323	34.0	63	0.73	4,3	8,2	10.50	91	
1/24	Diet: 50 grams cane sugar, 25 grams glucose											
2/2	806	1203	698	506	42.0	67	0.56	6,0	6,0	9.6	125	* Anis.++
2/9	763	1090	643	447	41.0	70	0.57	6,1	8,8	9.0	121	* Anis.++
2/14	750	1028	596	432	42.0	73	0.70	5,2	7,6	8.2	125	Dog refuses sugar

* Anisocytosis of red cells

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

Compare fasting period, table 7.

the hemorrhage, and this observation is in harmony with those of Buell (3) working with pigs. Our third experiment (table 17-a) shows little or no increase in urinary nitrogen, no clinical reaction and a low estimated blood volume (80 cc. per kgm.).

After the first exhibition of sugar we note a uniform low level of urinary nitrogen excretion. This low level is constant until the end of the experiments except in the first (table 15-a). Here we note a rise in urinary nitrogen during the last five days. It is fair to say that this

TABLE 15-A
Total urinary nitrogen—sugar. Dog 17-27

DATE, 1917	TOTAL NITRO- GEN 24 HOURS URINE	URINE TOTAL 24 HOURS	WEIGHT	REMARKS
	<i>grams</i>	<i>cc.</i>	<i>pounds</i>	
January 17	3.25	467	25.9	Fasting
18	2.58	411	26.0	
19	2.58	461	25.6	
20	2.86	422	25.1	Bled 375 cc.
21	3.75	381	24.1	0 feces
22	4.09	330	24.0	Bled 375 cc. Very weak
January 23	Anemia period begun—Diet: 50 grams sugar, 25 grams glucose			
23	3.30	537	23.8	0 feces
24	3.39	511	23.1	Diarrhea
25	1.99	427	22.8	0 feces
26	1.74	367	22.6	0 feces
27	2.02	390	22.3	Slight diarrhea
28	2.69	430	22.2	0 feces
29	2.38	435	21.9	0 feces
30	2.63	404	21.9	0 feces
31	1.74	401	21.7	0 feces
February 1	1.62	417	21.6	0 feces
2	1.46	401	21.2	0 feces
3	1.62	459	20.9	0 feces
4	1.96	375	0.8	Feces+
5	1.76	377	20.6	0 feces
6	2.07	400	20.5	0 feces
7	lost		20.4	0 feces
8	1.60	520	20.2	0 feces
9	1.90	475	19.8	Diarrhea
10	2.35	482	19.3	Diarrhea
11	3.86	720	18.4	0 feces. Vomitus +. Dog is sick
12	4.76	568	17.7	Vomitus +. Refuses sugar solution
13	4.76	361	18.1	0 feces
14	3.67	541	18.0	0 feces
15	4.20	505	17.8	Diarrhea +. Milk diet

Dog given 400 cc. water daily by stomach tube.

dog was sick, vomited the sugar solution and had diarrhea. This period is properly considered as an interval of intoxication in which little sugar was retained and perhaps this amount favored the intestinal irritation which was conspicuous.

TABLE 15-B

Experimental history. Dog 17-27

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 9/13/16 Bled 450 cc. End 10/24/16	Lean meat			<i>kgm.</i> 8.3 8.9 11.4	(3 bleedings) Complete regeneration of Hb. and R.B.C.
Begin 1/19/17 Bled 350 cc. End 2/16/17	Sugar, metabolism	1628 598 646	130 91 119	11.6 10.5 8.6	Table 15 Bread and milk 5 months. Slight regeneration
Begin 10/17/17 Bled 826 cc. End 11/24/17	Sugar, metabolism Sugar, gelatin, metabolism	2150 718 915	123 84 108	13.4 13.2 10.1	 Sugar, gelatin, crack- ermeal, lard and butter, 3 weeks. Dietary deficiency disease. Recovery 2 weeks
Begin 3/13/18 Bled 750 cc. End 4/10/18	Fasting	1626 1004 1240	72 72 93	15.7 15.5 10.7	Table 7
Begin 6/3/18 Bled 1022 cc. End 6/28/18	Cooked thymus, bread and milk	1939 723 1245	87 70 78	16.35 15.75 15.70	(3 bleedings)
Begin 8/9/18 Bled 988 cc. End 8/30/18	Hemoglobin intravenously. Sugar	1914 817 1155	90 67 80	16.15 15.45 13.75	Table 79 (3 bleedings) Killed September 3

Tables 18 and 18-b deserve special mention as they show the results of several experiments done under controlled conditions on the same dog. The experimental history refers to table 10, which shows the blood regeneration on this dog during a fasting period. There is a dis-

tinet increase in pigment volume, hematocrit and hemoglobin during the fasting period. We see in table 18 the same dog under identical conditions on a sugar diet. During the same interval we note practically the same value for pigment volume, hematocrit and hemoglobin at the beginning and end of the sugar feeding. There is a trifling rise during the first week but this is subsequently lost with return to the initial level. One of the tables to follow (table 21) shows the same dog on a sugar and

TABLE 16

Blood regeneration—sugar feeding—metabolism. Dog 17-28. White bull, female, adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/19	1620	1500	600	900	60.0	108	0.76	7,1	7,4	11.60	129	Fasting
1/20	Bled 375 cc.											
1/22	Bled 270 cc.											
1/23	Bled 105 cc.											
1/24	588	900	603	306	34.0	61	0.80	3,8	7,8	10.40	87	
1/24	Diet: 50 grams cane sugar, 25 grams glucose, 400 cc. water											
2/2	717	1121	684	437	39.0	64	0.62	5,2	7,2	9.40	120	* Anis.
2/9	636	1027	637	390	38.0	62	0.65	4,8	6,2	8.90	115	Diarrhea +
2/16	634	961	586	375	39.0	66	0.59	5,6	10,0	8.50	113	
2/23	541	933	562	373	40.0	58	0.56	5,2	9,0	8.00	117	

* Anisocytosis of red cells.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

Experimental history, see table 6-b.

gliadin diet which holds the pigment volume curve about on a level—no gain nor loss.

Table 19 is unsatisfactory from the standpoint of the blood volume and pigment volume figures but in view of the other experiments we venture to include it because this dog illustrates a reaction which is not uncommon in dogs bled for the first time. In dogs used for the first time in anemia experiments we often note a remarkable regeneration of

TABLE 16-A
Total urinary nitrogen—sugar. Dog 17-28

DATE, 1917	TOTAL NITROGEN 24 HOURS URINE	URINE TOTAL 24 HOURS	WEIGHT	REMARKS
	<i>grams</i>	<i>cc.</i>	<i>pounds</i>	
January 17	2.46	482	25.9	Fasting
18	2.10	340	25.8	
19	2.46	425	25.6	Diarrhea
20	2.21	519	24.8	Bled 373 cc.
21	2.80	375	23.6	
22	3.08	320	23.6	Bled 270 cc. Dyspnoea
23	2.49	493	23.6	Bled 105 cc.
January 24	Anemia period begun. Diet: 50 grams sugar, 25 grams glucose			
24	2.46	495	22.8	
25	1.96	470	22.4	0 feces
26	1.99	432	22.3	0 feces
27	2.86	427	22.1	0 feces
28	2.69	440	21.8	0 feces
29	2.07	446	21.5	Diarrhea +
30	2.04	406	21.3	0 feces
31	1.71	398	21.2	0 feces
February 1	1.62	391	20.9	
2	1.79	396	20.6	Trace feces
3	1.60	452	20.4	0 feces
4	1.74	407	20.3	0 feces
5	1.68	395	20.1	0 feces
6	1.23	383	20.0	0 feces
7			19.9	0 feces
8	1.74	425	19.7	Feces +
9	1.48	398	19.6	
10	1.51	415	19.4	0 feces
11	1.43	356	19.3	0 feces
12	1.40	387	19.2	0 feces
13	1.48	394	19.1	0 feces
14	1.51	388	18.9	0 feces
15	1.40	391	18.8	0 feces
16	1.26	390	18.6	Diarrhea +
17	1.51	378	18.4	0 feces
18	1.15	390	18.3	0 feces
19	1.23	399	18.1	0 feces
20	1.40	385	18.1	0 feces
21	1.43	409	17.9	0 feces
22	1.20	384	17.8	0 feces
23	1.12	346	17.6	0 feces
24	1.32	374	17.5	

Dog given 400 cc. water daily by stomach tube.

red cells and hemoglobin on unfavorable diets. This was not understood at first but it may be stated as a fact to be explained or not. It is possible that this type of dog has a greater reserve stored in its body from which it can construct red cells and hemoglobin on demand but a dog which has been bled at various times has not this large reserve. At any rate, this dog was able to give a remarkable exhibition of regeneration of red cells and hemoglobin during a short period of sugar feeding. The red cell hematocrit rose from 26 per cent to 47 per cent and

TABLE 17

Blood regeneration—sugar feeding—metabolism—splenectomy. Dog 17-34. White bull mongrel, female, adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/19	886	883	459	424	48.0	103	0.69	7,3	14,8	10.9	81	Fasting
1/20	Bled 221 cc.											
1/22	Bled 221 cc.											
1/24	414	780	546	234	30.0	53	0.80	3,3	11,4	10.1	77	
1/24	Diet: 50 grams cane sugar, 25 grams glucose, 400 cc. water											
2/2	528	979	676	303	31.0	54	0.60	4,5	6,4	9.3	105	* Anis.
2/9	536	893	607	286	32.0	60	0.62	4,8	6,2	8.8	101	
2/16	497	888	604	284	32.0	56	0.57	4,9	7,8	8.3	107	
2/23	556	868	567	304	35.0	64	0.60	5,3	6,4	7.8	113	

* Anisocytosis of red cells.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

hemoglobin from 62 to 121 per cent. This regeneration was almost completed during 4 weeks on a diet which is least favorable to blood regeneration.

Tables 20, 20-a and 20-b present data on sugar feeding which support the other experiments given above. This dog was observed during a fasting period (table 9) as well as during this sugar period and subsequently during other diet periods of hemoglobin regeneration. This experiment shows only a 2-week period of pure sugar feeding during

TABLE 17-A
Total urinary nitrogen—sugar. Dog 17-34

DATE, 1917	TOTAL NITRO- GEN 24 HOURS URINE	URINE TOTAL 24 HOURS	WEIGHT	REMARKS
	<i>grams</i>	<i>cc.</i>	<i>pounds</i>	
January 17	3.42	303	25.0	Fasting
18	3.30	424	24.4	Diarrhea +
19	2.63	406	24.0	
20	2.52	412	23.6	0 feces. Bled 221 cc.
21	2.58	326	22.9	
22	2.86	316	22.8	Bled 221 cc.
January 23	Anemia period begun. Diet: 50 grams sugar, 25 grams glucose			
23	2.66	526	22.8	Trace soft feces
24	2.35	436	22.2	
25	1.99	371	22.0	Trace feces
26			21.8	0 feces
27	2.46	351	21.6	Slight diarrhea
28	2.69	431	21.3	0 feces
29	2.60	371	21.2	0 feces
30	2.77	397	21.0	Trace of feces
31	1.68	382	20.8	0 feces
February 1	1.54	396	20.6	0 feces
2	1.46	396	20.4	
3	1.74	381	20.2	0 feces
4	1.63	426	20.0	0 feces
5	1.54	341	20.4	0 feces
6	1.51	406	19.8	0 feces
7			19.6	Diarrhea +
8	1.51	420	19.6	0 feces
9	1.48	406	19.3	
10	1.34	386	19.0	0 feces
11	1.51	380	18.8	0 feces
12	1.34	361	18.9	0 feces
13	1.60	412	18.7	0 feces
14	1.40	401	18.4	Trace of feces
15	1.40	381	18.4	0 feces
16	1.40	386	18.3	0 feces
17	1.37	396	18.0	Trace of feces
18	1.46	376	17.8	Trace of feces
19	1.46	432	17.7	0 feces
20	1.48	367	17.7	Trace of feces
21	1.34	411	17.6	0 feces
22	1.48	391	17.4	0 feces
23	1.62	431	17.1	0 feces
24	1.74	384	17.13	Diarrhea. Boiled milk diet

Dog given 400 cc. water daily by stomach tube.

TABLE 17-B
Experimental history. Dog 17-34. Splenectomy

EXPERIMENT NUMBER	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 12/15/16 Bled 390 cc. End 1/11/17	Meat and bread only	972	85	<i>kgm.</i> 10.9 11.3 12.5	No blood volume data Complete regenera- tion of Hb. and R. B. C.
Begin 1/20/17 Bled 442 cc. End 2/23/17	Sugar. Metabolism	910 413 556	81 77 113	10.9 10.1 7.8	Table 17 Bread and milk diet 5 months
Begin 6/3/18 Bled 807 cc. End 6/28/18	Powdered liver, bread and milk	1331 664 960	63 62 70	16.6 15.7 15.3	(3 bleedings)

Splenectomy 10/3/16.

TABLE 18
*Blood regeneration—sugar feeding. Dog 16-160. Bull mongrel, female, age 2
years +*

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
8/28	1835	1103	456	654	58.2	166	1.1	7,6	9,6	10.15	109	* Slight poik.
8/28	Diet: Crackermeal and milk											
8/28	Bled 276 cc.											
8/29	Bled 276 cc.											
8/31	648	771	524	243	31.5	84				9.55	81	
9/1	Bled 193 cc.											
9/3	448	696	513	177	25.5	64	0.8	4,0	15,4	9.5	73	* Poik.++
9/3	Diet: 75 grams sugar, 25 grams dextrose by stomach tube											
9/10	561	792	577	211	26.7	71	0.96	3,7	9,4	8.65	92	* Poik.++
9/16	488	703	500	192	27.3	69	0.78	4,4	7,8	8.0	88	* Poik.++
9/25	429	664	474	182	27.4	64	0.61	5,2	9,6	7.35	90	* Poik.++

* Poikilocytosis of red cells.

For continuation of experiment see table 80.

TABLE 18-B
Experimental history. Dog 16-160

EXPERIMENT NUMBER	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 9/26/16	Mixed			<i>kgm.</i> 5.70	No blood volume data
Bled 440 cc.				6.10	(4 bleedings)
End 11/29/16				7.30	Complete regen- eration of Hb. and R. B. C.
Begin 2/12/17	Bread, milk and Blaud's pills	938	127	7.70	Table 68
Bled 488 cc.		423	80	7.40	
End 7/16/17		456	97	5.90	Maximum regen- eration 17 weeks
Begin 10/17/17	Sugar and glia- din	1278	122	8.90	Metabolism
Bled 542 cc.		599	95	8.90	Table 21
End 12/3/17		755	106	6.30	Crackermeal, gel- atin, lard, but- ter, 2 weeks. Mixed diet 5 weeks. Com- plete regenera- tion
Begin 5/20/18	Fasting	1316	106	10.60	Metabolism
Bled 562 cc.		482	74	10.20	Table 10
End 6/12/18		636	94	7.55	Bread and milk 3 weeks
Begin 8/28/19	Sugar 3 weeks. Sugar and Hb. intravenously 1 week	1835	109	10.15	Table 18
Bled 745 cc.		448	73	9.50	(3 bleedings)
End 10/16/19		586	95	7.80	Dried yeast and crackermeal, 2 weeks. Slight regeneration. Table 80
Begin 2/20/19	Sugar and carrot juice Dried yeast, bread and milk Beef liver, bread and milk	1375	99	11.30	Table 22
Bled 781 cc.		548	81	10.10	(3 bleedings)
End 3/18/19		474	86	8.75	
Begin 8/8/19	Beet tops, bread and milk Spinach, bread and milk Compressed yeast, spin- ach, bread and milk	1220	97	10.80	
Bled 526 cc.		600	66	13.15	
End 12/5/19		799	95	8.60	

which time is recorded a moderate increase in red cells and pigment volume. This increase is somewhat above the average reaction under similar conditions and if sugar feeding had been continued for 2 more weeks a drop in hemoglobin, pigment volume and red cell hematocrit was to be expected.

Histidine (1.5 gram per day) added to this diet actually prevented the expected fall and is responsible for a *definite gain* in the first week of

TABLE 19

Blood regeneration—sugar feeding. Dog 19–28. Fox terrier, female, adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc	cc.	cc.	per cent	per cent				kgm.	cc.	
9/18	1193	790	341	441	55.8	151	0.94	8,0	8,4	8.35	94	
9/18	Diet: Crackermeal and milk											
9/20	Bled 198 cc.											
9/21	Bled 178 cc. Dyspnoea											
9/23	316	509	370	137	26.9	62	0.80	3,9	22,6	7.85	65	
9/23	Diet: 50 grams sugar daily with 150 cc. water											
10/2	637	637	349	284	44.5	100	0.81	6,2	8,6	6.90	92	
10/11					47.2	107				6.10		
10/18	562	464	242	219	47.3	121	0.89	6,8	7,8	5.75	81	*

* Hemolysis in tubes containing blood and dye. Reading of color unsatisfactory.

No previous anemia experiments on this dog.

histidine feeding. In view of the constancy of the sugar feeding reaction in the third week of blood regeneration we attach considerable importance to this reaction. We feel that histidine may be in part concerned in the complicated endogenous reaction which is responsible for the final elaboration of the complex protein hemoglobin.

The last 2 weeks of histidine feeding show merely a level curve of hemoglobin and pigment volume indicating that the maintenance factor alone is being supplied. Under sugar feeding alone a slowly fall-

ing curve would be expected, so that the reaction as a whole is strongly in favor of the histidine feeding as being a contributory factor in the hemoglobin regeneration under these experimental conditions. The small amount of histidine given is significant when we consider the percentage content of histidine in casein (2.5 per cent). It will be noted in a subsequent paper that casein is not an efficient food for hemoglobin regeneration.

TABLE 20

Blood regeneration—sugar and histidine—metabolism. Dog 17-38. White bull mongrel, female, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
9/11	1342	1231	505	726	59.0	109	0.59	9,3	14,0	11.60	106
9/11	Diet: Bread and milk										
9/12	Bled 308 cc.										
9/13	Bled 308 cc.										
9/15	468	884	619	265	30.0	53	0.68	3,9	12,8	11.30	78
9/15	Diet: 50 grams cane sugar, 25 grams dextrose, 300 cc. water										
9/21	572	805	550	290	36.0	71	0.71	5,0	11,8	10.30	78
9/28	574	755	468	287	38.0	76	0.58	6,5	6,6	9.60	79
10/1	Diet: 50 grams cane sugar, 25 grams dextrose, 1½ gram histidine, 300 cc. water										
10/5	668	795	468	326	41.0	84	0.68	6,2	8,4	8.90	76
10/12	735	826	471	355	43.0	89	0.58	7,7	7,2	8.00	103
10/19	684	786	456	330	42.0	87	0.60	7,3	9,2	7.30	107

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

The total nitrogen elimination is uniform and sustained at the expected low level except during the last week of the experiment when a decided increase is noted. This type of intoxication is not infrequent after long sugar diet periods and we believe is of gastro-intestinal origin. It is a fact that the hemoglobin and pigment volume show no increase during this period of increased protein katabolism but rather

TABLE 20-A

Total urinary nitrogen—sugar and histidine. Dog 17-38

DATE, 1917	TOTAL NITRO- GEN 24 HOURS URINE	URINE TOTAL 24 HOURS	WEIGHT	REMARKS
September 15	Diet: 50 grams cane sugar, 25 grams dextrose, 300 cc. water			
	<i>grams</i>	<i>cc.</i>	<i>pounds</i>	
16	2.52	415	23.9	Diarrhea. Vomited 55 cc.
17	1.93	370	23.7	Diarrhea. Vomited
18	1.46	401	23.4	Soft feces. Vomited 100 cc.
19	1.40	315	23.2	Vomited 50 cc.
20	1.34	240	23.1	0 feces
21	1.65	290	22.5	Diarrhea
22	1.23	285	22.5	0 feces
23	1.34	346	22.1	Slight diarrhea
24	1.34	247	21.8	0 feces
25	1.28	271	21.8	Slight diarrhea. Vomited
26	1.40	275	21.4	0 feces. Vomited 45 cc.
27	1.29	225	21.4	Soft feces. Vomited 25 cc.
28	1.20	245	21.2	Slight diarrhea
29	1.29	336	20.8	Soft feces
30	1.29	251	20.7	0 feces
October 1	Diet: 50 grams cane sugar, 25 grams dextrose, 1.5 gram histidine, 300 cc. water			
October 1	1.15	317	20.5	Feces +
2	1.23	290	20.5	0 feces
3	1.57	303	20.2	Slight diarrhea
4	1.34	264	19.9	0 feces. Vomited
5	1.65		19.6	
6	1.48	152	19.5	0 feces. Vomited 55 cc.
7	1.62	620	18.6	0 feces. Vomited
8	1.85	416	18.3	0 feces. Vomited
9	1.68	111	18.6	
10	2.63	401	18.1	0 feces
11	2.35	261	18.3	Vomited
12	3.25	453	17.6	0 feces. Vomited
13	3.08	341	17.6	0 feces
14	3.14	300	17.4	0 feces
15	2.86	415	17.1	0 feces
16	2.88	266	16.9	0 feces
17	2.58	329	16.8	0 feces
18	2.24	308	16.3	Moderate diarrhea
19	1.68	281	16.1	Diarrhea. Fair condition

TABLE 20-B

Experimental history. Dog 17-38

EXPERIMENT NUMBER	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 12/18/16	Sugar and gela- tin			<i>kgm.</i> 8.60	No blood volume data
Bled 600 cc.				8.20	(4 bleedings)
End 1/11/17		532	93	7.00	Slight regenera- tion of Hb. and R. B. C.
Begin 3/26/17	Fasting, metab- olism	1395	111	9.80	Table 9
Bled 544 cc.		443	79	9.30	
End 4/20/17		585	119	6.30	Bread and milk 3 months
Begin 9/11/17	Sugar Sugar and histi- dine Metabolism	1341	106	11.60	Table 20
Bled 616 cc.		468	78	11.30	
End 10/19/17		684	107	7.30	Beef heart fol- lowed by mixed diet
Begin 6/3/18	Gelatin, bread and milk	1621	86	12.15	
Bled 753 cc.		723	76	11.25	(3 bleedings)
End 6/28/18		1071	85	10.70	Mixed diet
Begin 8/8/18	Cooked brain, crackernel and milk	2060	109	11.65	
Bled 825 cc.		526	73	11.20	(3 bleedings)
End 9/27/18		1100	84	11.95	Pregnant Maximum regen- eration 3 weeks
Begin 2/6/19	Bread (343 grams) milk (200 cc.)	1873	117	12.15	Table 26
Bled 710 cc.		520	77	11.10	
End 3/19/19		1092	95	11.35	Maximum regen- eration 3 weeks
Begin 3/31/19	Bread (100 grams) milk (500 cc.)	1275	96	12.00	Table 26
Bled 580 cc.		499	78	11.40	
End 4/9/19		691	91	10.00	Maximum regen- eration 4 weeks

a slight loss in red cell hematocrit and pigment volume. This will be shown to be true in other *abnormal conditions* in which increased protein katabolism and urinary nitrogen excretion are observed.

TABLE 21

Blood regeneration—sugar and gliadin—metabolism. Dog 16-160. White bull mongrel, female, young adult

DATE, 1917	PIGMENT VOLUME = Hb PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
10/17	1397	1083	509	627	53.0	118	0.74	8,0	8,2	8.90	122	
10/17	Diet: Bread and milk											
10/19	Bled 271 cc.											
10/20	Bled 271 cc.											
10/22	600	844	574	270	32.0	71	1.04	3,4	11,4	8.90	95	
10/22	Diet: 75 grams cane sugar, 25 grams glucose, 300 cc. water											
10/29	617	812	552	260	32.0	76	0.88	4,3	8,2	8.0	101	*
11/5	770	794	548	246	31.0	97	0.87	5,6	6,0	7.4	105	*
11/5	Diet: 75 grams cane sugar, 25 grams glucose, 20 grams gliadin, 300 cc. water											
11/12	762	838	536	302	36.0	91	0.78	5,8	3,6	7.10	118	*
11/18	764	813	496	317	39.0	94	0.82	5,7	7,2	6.70	121	*
11/26	780	777	466	311	40.0	103	0.76	6,6	4,4	6.40	106	*
12/3	755	770	485	285	37.0	98	0.71	6,9	3,6	6.30	106	*

* Poikilocytosis and anisocytosis of red cells.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

Gliadin prepared in the usual way by dilute alcohol extraction of wheat flour. Experimental history, see table 18-b.

Tables 21 and 21-a give more data on sugar feeding periods. The hemoglobin regeneration is rather more marked than in the average experiment during a 2-week period. We note again a remarkable increase in red cells (2,000,000) and hemoglobin (26 per cent) with a stationary red cell hematocrit (32 per cent). If this observation had not been recorded in other experiments we might suspect an error.

TABLE 21-A
Total urinary nitrogen—sugar and gliadin. Dog 16-160

DATE, 1917	TOTAL NITRO- GEN 24 HOURS URINE	URINE TOTAL 24 HOURS	WEIGHT	REMARKS
October 22	Diet: 75 grams cane sugar, 25 grams glucose, 300 cc. water daily			
	<i>grams</i>	<i>cc.</i>	<i>pounds</i>	
23	2.77	413	18.6	0 feces
24	2.02	267	18.3	Solid feces
25	1.74	232	18.2	0 feces
26	1.46	178	17.8	Trace of feces
27	1.48	101	17.8	0 feces
28	1.62	251	17.8	0 feces
29	1.74	261	17.6	0 feces
30	2.10	256	17.3	Diarrhea +
31	1.43	217	17.5	0 feces. Vomited
November 1	1.51	131	16.8	0 feces
2	1.57	241	16.8	0 feces
3	1.46	471	16.0	0 feces
4	1.79	151	16.3	0 feces
November 5	Diet: 75 grams cane sugar, 25 grams glucose, 20 grams gliadin, 300 cc. water daily			
5	1.71	301	16.2	Trace feces
6	3.16	241	16.1	0 feces
7	3.53	192	15.9	Feces +
8	3.47	186	15.9	Diarrhea +
9	3.25	173	15.8	Diarrhea +
10	3.58	311	15.5	Diarrhea ++
11	3.25	277	15.4	0 feces
12	3.33	322	15.6	0 feces
13	3.47	301	15.3	Solid feces
14	3.53	319	15.1	Diarrhea +
15	3.50	156	15.1	Trace of feces
16	3.30	132	15.0	0 feces
17	3.25	176	14.9	Soft feces
18	3.30	151	14.7	Trace of feces
19	3.36	192	14.8	Diarrhea +
20	3.25	161	14.6	0 feces
21	3.36	156	14.5	Soft feces
22	3.22	151	14.4	Solid feces
23	3.58	156	14.4	Soft feces
24	3.47	182	14.3	Soft feces
25	3.36	181	14.2	Soft feces
26	3.33	173	14.1	Diarrhea +
27	3.47	222	13.9	Soft feces
28	3.22	152	14.0	Trace of feces
29	3.36	181	13.9	0 feces
30	3.08	168	13.9	Soft feces
December 1	3.70	166	13.9	0 feces
2	3.28	181	13.8	Trace of feces
3	3.42	163	13.8	0 feces

TABLE 22

*Blood regeneration—sugar plus carrot extract—yeast, bread and milk. Dog 16-160.
White mongrel, female, adult*

DATE, 1919	PIGMENT VOLUME Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
	cc.	cc.	cc.	per cent	per cent					kgm.	cc.	
2/20	1375	1114	508	601	53.9	123	0.70	8.8	6.0	11.30	99	
2/20	Diet: Bread and milk											
2/21	Bled 280 cc.											
2/22	Bled 280 cc.											
2/24	690	884	473	307	34.7	78	0.85	4.6	19.2	10.45	85	
2/24	Diet: 100 grams sugar, 100 cc. carrot juice, † 150 cc. water											
2/27	548	816	569	242	29.7	67	0.90	3.7	12.8	10.10	81	
3/5	500	818	568	238	29.1	61	0.71	4.3	11.6	9.65	85	
3/12	444	776	555	206	26.5	57	0.73	3.9	5.2	9.15	85	* Poik.++
3/18	474	750	530	211	28.2	63	0.79	4.0	6.8	8.75	86	* Poik.++ Shadow cells
3/18	Diet: 200 grams bread, 500 cc. milk											
3/26	520	812	575	232	28.6	64	0.71	4.5	7.4	9.20	88	* Poik.++
3/26	Diet: 200 grams bread, 3 grams yeast, 300 cc. milk											
4/2	516	816	556	251	30.8	63	0.63	5.0	6.2	9.45	86	* Poik.++
4/8	433	761	542	211	27.7	57	0.54	5.3	9.4	9.35	81	* Poik.++
4/14	416	726	517	197	27.5	57	0.57	5.0	7.2	9.30	78	* Poik.
4/21	535	828	553	275	31.2	65	0.59	5.5	9.4	9.40	88	
4/21	Diet: 200 grams cooked beef liver, 200 grams bread, 300 cc. milk											
4/28	631	851	542	305	35.8	74	0.70	5.3	20.4	10.30	83	R. B. C. fairly normal
5/7	1087	1055	548	496	47.0	103	0.71	7.3	10.4	10.70	99	
5/12	1153	1082	556	517	47.6	107	0.69	7.8	16.2	10.85	100	

* Poikilocytosis of red cells.

† Carrot juice = water extract of cooked carrots filtered and concentrated to one-third of its original volume.

Experimental history, see table 18-b.

This shows the possible fluctuation in hemoglobin content and size of red corpuscles which may be observed with a constant hematocrit. Probably many factors enter into this reaction which will be taken up again. The clinical summary of this dog shows a variety of diet periods including a fasting period (table 18-a).

Gliadin (20 grams per day) added to the sugar diet causes no increase in hemoglobin but merely a uniform maintenance factor. There is still further increase in red cells with poikilocytosis and we believe the evidence favors some red cell fragmentation under these conditions. The urinary nitrogen shows a uniformly low level during the sugar period and the expected level during gliadin feeding (table 21-a).

Table 22 gives the results of a second sugar regeneration period on the same dog (16-160) used in the preceding experiment (table 21). It will be observed that this sugar regeneration period is not as favorable and there is actually a loss in pigment volume amounting to about 30 per cent during a period of 4 weeks. This experiment includes another factor (carrot juice) which is obviously inert under these experimental conditions. This point will come up again in subsequent papers dealing with pigment derivatives. We wish to point out an unusual condition on February 24 after the 2 bleeding days when a low plasma volume (473 cc.) is recorded against the normal during the entire experiment of 500 to 550 cc. When the plasma volume returns to normal we note a fall in hemoglobin and red cell hematocrit. It is probable that the reaction is the result of the shock of the hemorrhage which is usually adjusted during the resting day intervening between the second bleeding and the second blood volume determination.

The second period of bread and milk feeding is included to substantiate the plasma volume figures. This reaction will be discussed in detail in the next paper.

Table 23 is given at the end of the series because one important factor separates it from all the other experiments,—*a bile fistula*. This dog (15-22) has been under observation in this laboratory for several years and many reports on bile excretion include experiments on this animal (4). It is known that traces of bile can gain entrance into this dog's intestine but the general condition of the dog is perfect. The reaction to anemia under fasting conditions in this dog is of particular interest as the absorption of pigments from the intestine may be excluded. It has been suggested by Addis (5) that absorption of some pigment complex from the intestine is a part of the body conservation of pigment materials. This and other experiments give no support to this interesting suggestion.

Bile pigment figures in a fasting period are given for this same dog in another publication (6). The constant presence of *urobilin* in the fasting bile of this experiment is noted in table 23 and discussed below. Total urinary nitrogen figures are not given here but were obtained in this experiment and average 3.08 grams per 24 hours. This is an average figure for a normal dog of similar weight and activity. Evidently

TABLE 23

Blood regeneration—fasting—metabolism—bile fistula. Dog 15-22. Brindle bull, male, age 4 years +

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
3/26	2200	1833	770	1063	58.0	120	0.77	7,8	12.6	15.40	119
3/26	Diet: Bread and milk										
3/28	Bled 460 cc.										
3/29	Bled 460 cc.										
3/30	673	962	654	308	32.0	70	0.83	4,2	18,6	15.10	63
3/30	Fasting begun										
4/2	890	1186	747	439	37.0	75	0.66	5,7	28,2	14.00	85
4/9	874	1136	625	511	45.0	77	0.70	5,5	14,6	12.50	91
4/16	1122	1069	556	513	48.0	105	0.71	6,4	6,2	11.40	94
4/23	1133	1059	540	519	49.0	107	0.66	8,1	7,2	10.40	116

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

No previous anemia experiments on this dog.

Urobilin constantly present in bile after 1st day of fasting period.

Total nitrogen in urine—average 3.08 grams per 24 hours.

the bile fistula does not modify the total nitrogen figures in this experiment nor in many others.

The curve of hemoglobin and pigment volume is quite remarkable. There is even more marked a new formation of red cells and hemoglobin than is noted in an average normal dog. We must not forget the occasional abnormal regeneration which is noted in a normal dog which has never been bled—a reserve being forthcoming which returns the

hemoglobin almost to normal in 3 weeks of fasting. This bile fistula dog had never been bled in any large amounts before. But the reserve reaction in this dog is truly remarkable and at the end of the fasting period we note a normal red count, a hemoglobin and red cell hematocrit figure close to normal and a considerable increase in pigment volume. It is noted that the plasma volume shows a decrease during the fasting period which gives the reason for the relatively low pigment volume. At any rate the capacity of this bile fistula dog for hemoglobin regeneration is in no way impaired and *may* be greater than normal.

TABLE 24
Fasting summary

DOG NUMBER	AFTER BLEEDING			TWO WEEKS LATER			THREE WEEKS LATER			FOUR WEEKS LATER		
	Pigment vol- ume	Hematocrit R. B. C.	Hemoglobin	Pigment vol- ume	Hematocrit R. B. C.	Hemoglobin	Pigment vol- ume	Hematocrit R. B. C.	Hemoglobin	Pigment vol- ume	Hematocrit R. B. C.	Hemoglobin
15-22	673	32.0	70	874	45.0	77	1122	48.0	105	1133	49.0	107
18-103	590	25.3	62	852	36.4	97	745	35.5	96	700	37.7	89
18-114	620	30.1	62	899	38.7	93	1103	44.4	102			
18-116	778	28.5	62	1025	36.0	85	1176	40.7	95			
17-38	443	28.0	60	491	39.0	68	585	43.0	76	585	44.0	78
17-37	313	28.0	50	492	35.0	64	532	35.0	73	540	35.0	70
16-160	482	33.2	64	591	33.5	88	636	34.3	90			
17-28	631	30.8	66	1007	43.8	102						
17-27	1005	31.0	90	1086	39.0	101	1213	41.0	115	1240	38.0	124
Fasting average.....	615	29.65	65	813	38.5	86	889	40.23	94	840	40.74	94
Sugar average, table 25.....	515	30.87	63	648	35.5	75	593	36.4	76	614	37.04	74

For example, we know that the bile fistula liver contains more pigment as demonstrated by the microscope. There may be stored away in the liver or other tissues more of the pigment complex from which the pigment reserve is derived—to be turned into finished hemoglobin on emergency demand.

The last three tables (tables 24, 25 and 25-a) give the summary figures of all the experiments in this paper and one fact stands out clearly in table 25-a. The *average blood regeneration* is distinctly greater during a fasting period of 2 weeks than during a similar sugar period of 2

weeks. The difference is even more striking at the end of 3 weeks when the average gain in pigment volume during fasting is 274, contrasted with 78 on sugar feeding. Hemoglobin figures show an average gain of 29 per cent on fasting contrasted with 13 per cent on sugar diet.

TABLE 25
Sugar diet summary

DOG NUMBER	AFTER BLEEDING			TWO WEEKS LATER			THREE WEEKS LATER			FOUR WEEKS LATER		
	Pigment volume	Hematocrit R. B. C.	Hemoglobin	Pigment volume	Hematocrit R. B. C.	Hemoglobin	Pigment volume	Hematocrit R. B. C.	Hemoglobin	Pigment volume	Hematocrit R. B. C.	Hemoglobin
17-28	588	34.0	61	636	38.0	62	634	39.0	66	541	40.0	58
17-34	414	30.0	53	536	32.0	60	497	32.0	56	556	35.0	64
17-38	468	30.0	53	574	38.0	76	668	41.0	84	735	43.0	89
16-160	600	32.0	71	770	31.0	97	762	36.0	91	764	39.0	94
16-160	690	34.7	78	500	29.1	61	444	26.5	57	474	28.2	63
19-28	316	26.9	62	916	47.2	107	562	47.3	121			
17-27	598	34.0	63	763	41.0	70	750	42.0	73			
16-160	448	25.5	64	488	27.3	69	429	27.4	64			
Sugar average.....	515	30.87	63	648	35.5	75	593	36.4	76	614	37.04	74
Fasting average, table 24.....	615	29.65	65	813	38.5	86	889	40.23	94	840	40.74	94

TABLE 25-A
Gains made above minimum level after bleeding

	DURING TWO WEEKS (TOTAL)			DURING THREE WEEKS (TOTAL)		
	Pigment volume	Hematocrit R. B. C.	Hemoglobin	Pigment volume	Hematocrit R. B. C.	Hemoglobin
Nine experiments, fasting average.....	198	8.85	21	274	10.58	29
Eight experiments, sugar average.....	133	4.63	12	78	5.53	13

DISCUSSION

The term "sparing action of carbohydrates" is familiar to all workers in metabolism and means that carbohydrate feeding will decrease the excretion of total urinary nitrogen by man or animal as compared with the fasting excretion of nitrogen. It is therefore assumed that

the administration of carbohydrate actually spares the body protein. There are two theories for this sparing action of the carbohydrates: *a*, That the sparing action is at the source—that is, the sugar prevents tissue katabolism or spares protein in tissue cells; *b*, That the sparing action is a conservation of split products which, with the aid of carbohydrate radicles, are reconstructed into a variety of protein complexes and used in various parts of the body. Both theories have able advocates, but it is fair to say that most of the experiments can be used by a skilful proponent to support either hypothesis. An admirable review of the work in this field has been published recently by Janney (7).

Some experiments by Davis and Whipple (8) concerning the regeneration of liver cells following a unit injury can be said to support convincingly the theory of conservation of protein end products. In these experiments there is little liver cell repair during fasting periods but a rapid repair during sugar diet periods. This indicates that the body can form many new liver cells when sugar is available but not during a fasting period. These new liver cells must be formed from body protein split products, and this type of conservation of nitrogen is due to sugar feeding and cannot be explained by any amount of protein sparing at the source.

The experiments, summarized in tables 24, 25 and 25-a, follow the normal regeneration of another type of body cell: the normal red blood cell which is constantly being used up and reformed or reconstructed day by day. This is an admirable cell for a study of cell regeneration as its main constituent (hemoglobin) is very complex and easily and accurately measured. The amount of hemoglobin circulating in the body can be measured with considerable accuracy and therefore its curve of regeneration can be established with reasonable precision. When we compare periods of regeneration of hemoglobin during fasting and during sugar diet periods we find a constant difference which comes out clearly in an average figure of many experiments (table 25-a). This table shows that the fasting dog can regenerate more red cells and hemoglobin than a dog on a sugar diet. This figure is over and above the maintenance supply of hemoglobin which is needed to keep the level uniform and furnish the hemoglobin used up by the daily wear and tear of the circulation in the body.

This actual reconstruction of new red cells and hemoglobin must come from the body protein or its split products as no nitrogen is being supplied to the body. Evidently the body conserves very carefully the substances which are suitable for the elaboration of hemoglobin. The

pyrrol complex is a peculiar feature of the hemoglobin molecule which can scarcely be formed in the body and may be an important determining factor in its reconstruction. How may we explain the increase in hemoglobin during fasting periods in excess of the reaction on sugar feeding? This surely cannot be explained by increased synthetic capacity due to the presence of sugar or the conditions should be reversed. If we assume that sugar may have a certain sparing action at the source we are able to suggest a plausible explanation. Suppose the sugar feeding does protect body protein from katabolism and therefore lessens the amount of available protein split products, we are then able to explain the smaller amount of hemoglobin produced, *provided* we assume that under all circumstances of need or limited diet the body conserves *all* the available protein building stones which go to make up the hemoglobin molecule. This seems to us to be the best explanation of the observed facts, but this opinion may be modified by further work.

It may be objected that the "maintenance factor" of red cells may vary in fasting as compared with sugar periods. This factor is not to be determined at this time, but we have no reason to suppose that the daily wastage of red cells should be greater on sugar feeding than during fasting periods. This question must be left open for the present.

Granting the facts as outlined above we may say that we have good proof to explain the "sparing action of carbohydrates" as due to a conservation of protein split products which aid in new protein construction as observed in liver repair (8). But these anemia experiments may be best explained by a "sparing action of carbohydrate" which protects at the source the body protein from katabolism. If this work is correct we may assume that the carbohydrate in the diet may have a double "sparing action"—*to protect the body protein at its source and to aid materially in the conservation of protein split products*, which are recast into new body protein. That one or the other of these two reactions may be dominant under varying conditions may be granted as probable.

No discussion of any phase of pigment metabolism is complete without proper consideration of the pigment *output in the bile*. The bile pigment represents in part at least under certain conditions the end product of hemoglobin degradation in the body, but in addition under certain conditions this bile pigment may represent certain constructive activities of the liver (9). It is possible that a part of this bile pigment produced by the liver and not derived from the degradation of hemoglobin may be an excess of pigment substance available for hemoglobin *production* but not so used and later discarded by way of the bile. This

reaction might only take place in the liver when there was a considerable surplus of pigment elements in the food or elsewhere. Such conditions might not obtain in the body when it was deprived of all protein intake (fasting or sugar diet).

The bile pigment output, therefore, must be reviewed briefly in this place. We are able to refer to observations of this nature (6) and state that there may or may not be differences in the bile pigment excretion during fasting periods as compared with sugar feeding periods in bile fistula dogs. Certain experiments appear to show greater bile pigment elimination during sugar periods than during fasting periods but other experiments show little or no difference in the bile pigment figures. These differences may represent individual variations in these bile fistula dogs and the available data are not sufficient to establish any difference in bile pigment excretion under these conditions. One thing is quite clear—these bile fistula dogs during fasting or sugar periods do excrete a measurable amount of bile pigments (average of 15 to 30 mgm. bile pigment per 6 hour daily collection). This pigment results from the degradation of hemoglobin in the body or the production of pigment complex from other substances in the liver—in other words, a distinct loss of pigment material from the body.

One other point must be mentioned in this connection. During fasting periods in bile fistula dogs we have noted the invariable appearance of *urobilin in the bile*. In our experience this is the only condition which is constantly associated with urobilin production in the dog's liver. At times the bile pigments may be almost completely replaced by the urobilin pigment and this introduces a serious error in our analysis of bile pigment. It is probable (if not certain) that this urobilin is derived from the bile pigment and its increase therefore will be associated with a corresponding decrease in bile pigments. But we have no accurate quantitative analytical method for urobilin, although we can estimate bile pigments there present by precipitation of the calcium pigment compound, filtration and analysis of the acid alcohol derivative. It seems probable to us that the urobilin appearing in the bile fistula dogs is derived at least in part from the bile pigments in the bile ducts, due to the activity of bacteria which we know are responsible in part at least for this reaction in the intestine. During fasting periods the flow of bile is very sluggish and this inferior drainage of bile gives a favorable opportunity for the bacteria to multiply. It can be stated that bacteria are numerous in all bile fistula tracts and may at times set up an inflammatory reaction in the bile passages which will cause

trouble. Flushing out the bile passages by cholagogue action as a rule gives relief to this condition.

It has occurred to us that this observation may have some significance as regards urobilin in the urine in a variety of conditions. There is no conclusive proof that urobilin is ever absorbed from the intestine. We have here proof that urobilin may be formed in the liver. It would seem safe to assume that the *hepatic origin of urobilin* should be considered in any analysis of this complex question. When the possibility is suggested that *urobilin may be formed at times in the liver*, it is obvious how difficult it is to exclude this *possibility* in the clinical conditions associated with which we note urobilin in the urine.

SUMMARY

During *fasting periods* after unit hemorrhages the normal dog can regenerate measurable amounts of red cells and hemoglobin.

This regeneration of red cells and hemoglobin *includes* the daily wastage of these elements, or the maintenance factor of the blood. The curve of hemoglobin regeneration represents the production of hemoglobin *in excess* of this unknown maintenance factor.

Bile pigment excretion under fasting or sugar diet conditions may be considered as uniform. A bile fistula dog may regenerate hemoglobin and red cells with at least equal and perhaps greater speed than a normal dog. The constant presence of *urobilin* in the bile of the fasting bile fistula dog is recorded and discussed from the standpoint of *urobilinuria*. The hepatic origin of urobilin is suggested.

During *sugar diet periods* the regeneration of hemoglobin and red cells is *distinctly less than during fasting periods* (table 25-a).

We believe that this observation may be explained by a double "sparing action of carbohydrates"—both sparing at the source or protecting body protein from katabolism as well as effecting synthetically a distinct conservation of protein split products. This postulates a strict conservation by the body of certain protein fractions which may be recast into hemoglobin. The presence of carbohydrate may facilitate this reaction but the actual new formation of hemoglobin may depend in part upon the type and amount of amino acid groups available from normal protein katabolism.

Histidine given with sugar appears to cause a production of hemoglobin over the control level. This amino acid may be one of the important elements in this hemoglobin regeneration complex.

Gliadin in the amounts used does not modify the hemoglobin reaction.

BIBLIOGRAPHY

- (1) ASHBY: Journ. Exper. Med., 1919, xxix, 267.
- (2) SEIDELL: Public Health Repts., U. S. Public Health Service, no. 325, 1916, 364.
- (3) BUELL: Journ. Biol. Chem., 1919, xl, 62.
- (4) WHIPPLE AND HOOPER: This Journal, 1917, xlii, 256.
- (5) ADDIS: Arch. Int. Med., 1915, xv, 413.
- (6) FOSTER, HOOPER AND WHIPPLE: Jour. Biol. Chem., 1919, xxxviii, 393.
- (7) JANNEY: New York Med. Journ., 1918, cvii, 824, 879.
- (8) DAVIS AND WHIPPLE: Arch. Int. Med., 1919, xxiii, 689.
- (9) WHIPPLE AND HOOPER: This Journal, 1917, xliii, 258, 290.

BLOOD REGENERATION FOLLOWING SIMPLE ANEMIA

III. INFLUENCE OF BREAD AND MILK, CRACKERMEAL, RICE AND POTATO, CASEIN AND GLIADIN IN VARYING AMOUNTS AND COMBINATIONS

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Early in our work it became evident that bread and milk did not constitute a favorable diet for the rapid regeneration of blood. This diet is palatable to dogs and maintains them in good nutritional condition for many weeks. As a result we began to use this diet as a maintenance diet to which other factors could be added which did or did not modify the curve of blood regeneration to be expected from the bread and milk factors alone. Because in many experiments we use bread and milk as a part of the diet it is essential that we understand clearly the effect of this diet under a variety of conditions. We therefore submit many experiments tabulated below to establish the normal blood regeneration of the dog which is limited to varying amounts of dried white bread and skim milk.

We must mention in passing the dietary deficiency disease which develops in dogs kept for long periods on a strict bread and milk diet. This disease condition resembles scurvy in human beings and is rapidly fatal if not energetically treated by antiscorbutic measures. This condition will be reviewed in a subsequent publication.

It will be noted from the experiments here outlined that bread and milk alone when given in large amounts may return the blood picture to normal in six weeks or longer. But when given in moderate amounts (100 grams dried bread and 500 cc. skim milk) this diet will rarely permit of complete blood regeneration. On this diet the hemoglobin, pigment volume and red cell hematocrit may be kept at a permanently subnormal level following the unit hemorrhages used in our experiments to produce uncomplicated secondary anemia. The value of

establishing this fact is obvious when it is found that some food materials added to this diet will profoundly modify this reaction expected from bread and milk alone. These observations will be reported in subsequent communications.

A few experiments with two of the important constituents of the bread and milk diet are included. Casein and gliadin in sufficient amounts are able to modify somewhat the reaction expected from sugar feeding alone. Some work with incomplete proteins and mixtures of amino acids has been completed but the evidence, so far, is not conclusively in favor of any single amino acid as being responsible for this peculiar reaction which depends in great measure upon the capacity of the body to construct hemoglobin.

Crackermeal and milk were used at one period during the war when white bread or in fact any kind of bread was not available for obvious reasons. This crackermeal was purchased on the open market during the war period and its constitution is not accurately known. We were able to ascertain with reasonable certainty that this crackermeal contained 70 to 80 per cent wheat flour, but a considerable percentage of barley and rice flour. Other grains may possibly have been concerned. The fact remains that this mixture of wheat flour and other grain flours did not modify in any manner the reaction established recently for commercial white bread which at present is made almost wholly from wheat flour.

Rice, potatoes and milk are used in one large series of experiments. The amount of blood regeneration on this diet closely parallels that observed on a bread and milk diet. This diet also includes one other substance sometimes used in bread,—potato or potato flour. Any one of these three diets is a favorable maintenance diet to which other factors may be added to determine the value of the unknown substance in its relation to blood regeneration.

EXPERIMENTAL OBSERVATIONS

Unless otherwise noted, the same technique is used in these experiments which has been described above (paper I). The food mixtures were all palatable and readily eaten unless note is made to the contrary. With few exceptions the dogs maintained their weight, general activity and health throughout the experiments.

Bread and milk diet. The first experiment (table 26) in this series illustrates many points which are established by the succeeding experi-

ments given below. The dog presented a very high hemoglobin (130 per cent), high red count (10 million) and blood per kilo (127 to 117 cc.). Following the unit hemorrhages there is recorded as usual a volume of red cells (239 cc.) which is much below the calculated expected red cell volume (438 cc.). The low level in this experiment is more noticeable than in the average experiment. The plasma volume is promptly made up to normal after the bleedings and remains as usual relatively constant. The curve of regeneration is quite steep during the first 2 weeks of the bread and milk diet but thereafter remains at a uniform level. This statement applies to the pigment volume, red cell hematocrit, and hemoglobin, but the red cell count shows a slow increase toward normal in the last 3 weeks of the experiment.

In this first experiment the diet was abundant and sufficient to maintain the body weight and even to allow of slight increase. This point is of much importance, as will appear later. This and other similar experiments show that a *liberal bread and milk diet* sufficient to maintain or increase the body weight will cause a certain degree of hemoglobin regeneration over and above the daily maintenance hemoglobin factor. Blood regeneration may be rapid for a week or two and may even return the blood picture almost to normal in certain experiments.

The repeat experiment on this same dog (table 26) shows a lower initial hemoglobin and red count. The hemorrhages are less in amount but the anemia level for pigment volume is much the same as in the preceding anemia period. The diet now is not abundant and contains only 100 grams dried bread as compared with 343 grams in the first period of regeneration. This 100 grams bread diet is not sufficient to maintain the body weight at its normal level and there is a loss in weight of 1.4 kilos during the 5 weeks of blood regeneration. There is a striking difference in the amount of hemoglobin regeneration which shows only a trivial increase from week to week over the maintenance factor. In using the term *maintenance factor* we wish to indicate that unknown replacement fraction which represents the daily wastage of red cells used up in the body metabolism. There are experiments to indicate that this fraction may be 3 per cent per day in human beings, but there are no data to establish this important point for the dog.

It is obvious from these two anemia periods (table 26) that a dog will regenerate more hemoglobin on an abundant bread and milk diet than on a limited bread and milk diet. This applies particularly to the *bread* portion of the diet. The term "bread" as used in this

TABLE 26

Blood regeneration—bread and milk—repeat experiment. Dog 17-38. Bull mongrel, female, young adult

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/30	1910	1467	585	877	59.8	130				11.5	127	
2/6	1873	1418	600	812	57.2	132	0.66	10,0	9,0	12.15	117	
1/30	Diet: White bread and milk											
2/7	Bled 355 cc.											
2/8	Bled 355 cc.											
2/10	520	852	608	239	28.1	61	1.0	3,1	28,4	11.1	77	
2/10	Diet: Dried, ground white bread, 343 grams, skim milk, 500 cc.											
2/17	852	1074	654	404	37.6	79	0.88	4,5	12,0	11.6	93	
2/17	Diet: Dried, ground white bread, 343 grams, skim milk, 200 cc.											
2/26	1120	1133	588	534	47.1	99	0.72	6,9	11,8	11.6	98	
3/3	1137	1110	560	544	49.0	102	0.82	6,2	7,4	11.35	98	* Poik. +
3/10	1138	1083	575	497	45.9	105	0.72	7,3	7,8	11.5	94	* Poik. +
3/19	1092	1072	561	493	46.0	102	0.66	7,7	6,8	11.35	95	* Poik. +
3/21	Mixed diet											
3/31	1275	1155	558	586	50.7	110	0.67	8,2	7,2	12.0	96	* Poik. ++
3/31	Diet: White bread and milk											
4/1	Bled 290 cc.											
4/2	Bled 290 cc. No distress											
4/3	499	887	633	249	28.1	56	0.67	4,2	9,6	11.4	78	* Poik. +
4/3	Diet: Dried, ground white bread, 100 grams, skim milk, 500 cc.											
4/11	566	989	662	307	31.0	64	0.68	4,7	11,0	11.25	77	*
4/18	662	988	638	335	33.9	67	0.56	6,0	9,2	11.0	90	*
4/25	652	920	578	304	35.4	71	0.55	6,5	6,6	10.7	86	*
5/2	734	955	585	367	38.4	77	0.57	6,7	7,2	10.35	92	* Poik. ++
5/9	691	909	564	341	37.5	76	0.58	6,5	5,6	10.0	91	* Poik. ++

* Poikilocytosis of red cells.

Experimental history, see table 20-b.

TABLE 27

Blood regeneration—bread and milk—repeat experiment. Dog 19-94. Bull mongrel, male, age 5 months

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/16	1250	1343	790	540	40.2	93	0.57	8.1	21.6	10.65	126	
1/16	Diet: Bread and milk											
1/17	Bled 335 cc.											
1/18	Bled 285 cc. No distress											
1/20	462	839	645	190	22.6	55	0.95	2.9	14.6	10.00	84	
1/20	Diet: 250 grams dried, ground white bread, 500 cc. skim milk											
1/27	576	1020	683	327	32.0	56	0.55	5.1	11.0	10.25	100	*
2/3	900	1088	651	485	39.7	83	0.57	7.3	13.4	10.25	106	* Poik. +
2/12	750	1000	626	362	36.2	75	0.72	5.2	10.2	10.80	93	* Poik. +
2/19	907	1085	647	439	40.4	84	0.69	6.1	9.8	11.15	97	*
2/28	994	1126	667	448	39.8	88	0.76	5.8	6.2	11.50	98	*
3/7	899	1098	650	437	39.8	82	0.73	5.6	8.4	11.70	94	*
3/10	Diet: Mixed diet											
3/17	1146	1216	687	523	43.0	94	0.70	6.7	10.6	13.05	93	* Slight
3/17	Diet: White bread and milk											
3/18	Bled 304 cc.											
3/19	Bled 304 cc. No distress											
3/21	542	968	709	255	26.3	56	0.82	3.4	14.4	12.40	78	
3/21	Diet: 300 grams dried, ground white bread, 500 cc. skim milk											
3/28	736	979	640	324	33.1	75	0.89	4.2	12.6	12.50	94	* Slight
4/2	811	1112	712	395	35.5	73	0.78	4.7	8.4	12.45	89	*
4/9	864	1110	680	420	37.8	78	0.65	5.9	11.4	12.40	89	* Slight
4/16	1005	1200	718	469	39.1	84	0.70	6.0	8.0	12.75	94	* Slight
4/23	1094	1236	740	485	39.2	88	0.71	6.9	6.0	12.75	97	* Slight
4/30	1031	1311	800	498	38.0	79	0.55	7.2	10.0	13.20	99	* Slight

* Poikilocytosis of red cells.

No previous anemia experiments with this dog.

report indicates white bread of the first quality, obtained from the University Hospital, sorted, dried in an oven and pulverized.

Table 27 gives a repeat experiment which shows that the curve of blood regeneration following a very short resting period is practically identical under uniform diet conditions. It is of considerable importance to know beyond question whether we may expect a uniform reaction under uniform conditions when a dog is used for different experiments at different intervals of time. We believe this communication includes sufficient data to establish this point beyond question. Therefore we may feel secure in using for anemia work the same set of dogs, *provided* the blood picture has returned to normal and the weight and general health is also normal. With repeated anemia experiments the dog does not increase in its capacity to regenerate hemoglobin nor does this reparative mechanism fail under the conditions of these repeat experiments. We may then attach considerable significance to deviations from the standard reaction in any given animal.

The repeat experiment (table 27) gives a reaction curve of pigment volume, red cell hematocrit and hemoglobin which is practically identical with the first anemia period. There is slightly more gain in the repeat experiment than in the first anemia observation. In both anemia periods the diet was liberal and permitted a gain in body weight of approximately 1 kilo per 6-week period. The repeat experiment brought the hemoglobin back more nearly to normal but the initial anemia level was not as low nor was the loss by hemorrhage as great. In the repeat experiment the dog received 300 grams dried white bread in contrast to 250 grams bread in the first period, but this was only a proper proportion per kilo body weight. These dogs, moreover, were in a period of rapid body growth (5 to 8 months).

The anemia experiments given in table 28 are very similar to those just described. In this instance, too, the bread and milk diet was sufficient for maintenance plus a definite growth factor with a gain of 2 kilos in body weight during the 5-week periods. In both periods the regeneration brings the pigment volume and blood picture back to the normal level. The steady gain in weight and body growth must not be lost sight of in reviewing the same gain in plasma volume. In the adult normal dog the plasma volume is now known to be quite constant under these experimental conditions.

The experiment given in table 29 is slightly different from those preceding. After the anemia period we have 1 week's fast which shows as usual a definite gain in pigment volume. The subsequent 3 weeks

TABLE 28

Blood regeneration—bread and milk—repeat experiment. Dog 19-102. Bull mongrel, male, age 5 to 6 months

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/30	972	944	482	453	48.0	103						
2/6	1026	1068	606	452	42.3	96	0.63	7,6	16,0	10.90	98	
2/6	Diet: Bread and milk											
2/7	Bled 267 cc.											
2/8	Bled 267 cc. No distress											
2/10	418	803	594	205	25.5	52	0.79	3,3	11,4	10.25	78	
2/10	Diet: 283 grams dried, ground white bread, 500 cc. skim milk											
2/17	720	951	608	337	35.5	76	0.93	4,1	8,6	10.65	89	
2/26	888	1014	592	417	41.1	87	0.65	6,7	11,6	11.25	90	* Poik. +
3/3	1045	1068	575	488	45.7	98	0.76	6,4	12,8	11.35	94	* Poik. +
3/10	1020	1045	578	462	44.2	97	0.73	6,6	11,2	11.55	91	* Poik. ++
3/19	1026	1056	576	467	44.2	97	0.70	6,9	12,0	12.55	86	* Poik. ++
3/22	Diet: Mixed diet											
3/31	1220	1220	644	564	46.2	100	0.67	7,5	12,6	13.20	93	* Poik. ++
3/31	Diet: Bread and milk											
4/1	Bled 305 cc.											
4/2	Bled 305 cc. No distress											
4/3	470	874	634	232	26.5	54	0.68	4,0	16,8	12.50	70	* Poik. ++
4/3	Diet: 283 grams dried, ground white bread, 500 cc. skim milk											
4/11	758	1085	702	361	33.3	70	0.69	5,1	7,4	13.30	82	*
4/18	884	1083	650	421	38.9	82	0.64	6,4	12,8	13.50	80	
4/25	1099	1163	660	498	42.8	94	0.64	7,3	9,6	13.85	84	*
5/2	1648	1485	701	769	51.8	111	0.74	7,5	11,2	14.55	102	*
5/9	1280	1268	667	584	46.0	101	0.64	7,9	19,8	14.50	87	*

* Poikilocytosis of red cells.

No previous anemia experiments with this dog.

show a stationary pigment volume up to the last week, when there is a definite gain. This diet was sufficient for maintenance of body weight and the general condition was uniformly excellent.

TABLE 29

Blood regeneration—bread and milk. Dog 18-123. Brindle bull mongrel, male, young adult

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
5/1	1692	1270	546	716	56.9	133	0.68	9,8	12,2	12.55	101	
5/1	Diet: Bread and milk											
5/2	Bled 318 cc.											
5/3	Bled 318 cc. No distress											
5/5	592	826	552	270	32.7	72	0.64	5,6	18,8	11.60	71	
5/6	Bled 200 cc.											
5/8	440	668	459	203	30.3	66	0.85	3,9	12,0	10.90	61	
5/8	Fasting											
5/14	620	802	515	283	35.3	77	0.73	5,3	12,2	10.30	78	
5/14	Diet: 100 grams dried, ground white bread, 500 cc. skim milk											
5/21	638	852	560	270	31.7	75	0.73	5,1	17,0	9.95	96	* Poik. +
5/28	633	891	582	299	33.6	71	0.66	5,4	14,2	9.90	90	* Poik. +
6/4	798	929	574	345	37.2	86	0.63	6,8	10,6	9.75	95	* Poik. +

* Poikilocytosis of red cells.

No previous anemia experiments on this dog.

The experiments given in tables 30 and 31 are very similar and may be discussed together. In both experiments the amount of bread and milk was not measured, but it is significant that there was a slight but definite loss of body weight during the bread and milk periods. It is to be expected, therefore that the diet would not suffice to raise the level of hemoglobin and pigment volume much above the anemia

level. The larger dog (table 30) does show a slow regeneration toward normal in 4 weeks, but the smaller dog (table 31) shows almost no net gain during 3 weeks. There is a little gain in the first week which is lost subsequently.

The mixed diet reaction is very nicely shown in both experiments (tables 30 and 31) and a week or two is sufficient to make up the deficit in hemoglobin and establish the normal level.

TABLE 30

Blood regeneration—bread and milk. Dog 18-113. Bull mongrel, female, age 4 to 5 months

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
4/24	970	1000	561	426	42.6	97	0.63	7,7	9,6	9.75	103	
4/24	Diet: Bread and milk—amount not measured											
4/25	Bled 250 cc.											
4/26	Bled 170 cc. No distress											
4/29	418	853	559	277	32.5	49	0.52	4,7	12,6	9.50	90	
5/8	597	776	468	295	38.0	77	0.61	6,3	8,6	9.13	85	
5/15	878	1140	674	445	39.1	77	0.49	7,9	9,6	9.10	125	*Fragm.
5/22	666	912	555	341	37.3	73	0.52	7,0	10,2	9.30	98	*Fragm.
5/29	701	788	473	315	40.0	89	0.55	8,1	9,8	9.10	87	*Fragm.++
5/29	Diet: Mixed diet											
6/10	940	940	530	397	42.2	100	0.60	8,4	10,2	10.45	90	*Fragm.

* Fragmentation of red cells.

No previous anemia experiments on this dog.

A *bile fistula* experiment is included (table 32) to show that these dogs react like normal dogs as regards blood pigment production after simple anemia. This dog was known to have complete exclusion of bile from the intestinal tract (autopsy notes). His general condition during the entire experiment was excellent and the bread and milk diet was sufficient to maintain the body weight close to normal. During the entire period there was a loss of only 1.4 kilos, which is not

great when we consider the size of the dog and the length of the experiment (7 weeks). There was a slight initial gain in hemoglobin and pigment volume which was subsequently lost. The general level of pigment volume, red cell hematocrit and hemoglobin is pretty nearly uniform with occasional temporary gains and losses. It is of interest to note a steady gain in number of red cells from 3,400,000 to 6,600,000. The hemoglobin gain was much less, which gives a fall in color index

TABLE 31

Blood regeneration—bread and milk. Dog 18-115. Bull mongrel, female, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
4/24	655	780	394	368	47.2	84	0.59	7.1	14.2	7.00	111	
4/24	Diet: Bread and milk—amount not measured											
4/25	Bled 195 cc.											
4/26	Bled 140 cc.											
4/29	314	582	375	200	34.3	54	0.63	4.3	26.2	7.30	80	
5/8	572	752	467	274	36.45	76	0.59	6.5	9.4	6.77	111	
5/15	675	888	522	353	39.8	76	0.59	6.4	25.0	6.40	138	
5/22	468	593	354	233	39.3	79	0.70	5.6	6.0	6.20	96	
5/23	Diet: Mixed diet											
5/29	516	607	373	228	37.5	85	0.64	6.6	10.8	6.90	88	* Poik.
6/10	769	761	438	318	41.8	101	0.73	6.9	14.2	8.00	95	* Poik.

* Poikilocytosis of red cells.

No previous anemia experiments on this dog.

from 0.87 to 0.54. This is not uncommon in normal dogs under similar experimental conditions.

Crackermeal, milk, lard and butter. These crackermeal experiments were performed in part during the war period when white bread was not available. This crackermeal consisted of an unknown mixture including at least wheat, barley and rice flours, possibly others. The experiments show reactions which resemble accurately those observed

with white bread. A mixture of grain flours, therefore, is no more efficient in promoting a regeneration of hemoglobin and red cells than white bread alone, consisting mainly of wheat flour.

Table 33 is to be compared with table 30—the first experiment done with white bread and milk, and the second with crackermeal and milk.

TABLE 32

Blood regeneration—bread and milk diet—bile fistula. Dog 17-151. White bull mongrel, male, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
5/28	2675	2158	971	1085	55	124	0.72	8,6	5,2	18.20	113
5/28	Diet: Bread and milk										
5/29	Bled 540 cc.										
5/30	Bled 540 cc.										
5/31	830	1408	1000	408	29	59	0.87	3,4	14,6	17.50	80
5/31	Diet: Bread and milk—amount not measured										
6/8	1058	1557	1059	498	32	68	0.77	4,4	9,8	17.20	90
6/15	1575	1852	1222	630	34	85	0.65	6,5	6,6	16.80	110
6/22	1274	1464	937	527	36	87	0.63	6,9	10,8	16.40	89
6/29	990	1415	920	495	35	70	0.49	7,2	7,4	16.10	88
7/6	1150	1532	950	582	38	75	0.54	6,9	5,8	15.90	96
7/13	1480	1741	1097	644	37	85	0.55	7,7	13,8	15.90	109
7/18	1118	1574	1039	545	34	71	0.54	6,6	13,8	16.10	98

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

No previous anemia experiments on this dog.

These experiments were performed during a period of rapid growth and the diets in each instance were sufficient to preserve body weight. There was a slight gain in weight during the crackermeal experiment. Both experiments show a slow steady gain in pigment volume, hemoglobin and red cell hematocrit. We may say the curves are as nearly identical as one can hope to observe in this type of experiment.

After a period of 8 weeks of crackermeal and milk diet the dog suddenly developed acute *dietary deficiency disease* which resulted in

TABLE 33

Blood regeneration—crackermeal and milk. Dog 18-113. Bull mongrel, female, age 8 to 9 months

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
8/14	1556	1136	571	557	49.0	137	0.81	8,4	5,2	12.85	88	
8/15	Diet: Bread and milk											
8/16	Bled 284 cc.											
8/17	Bled 284 cc.											
8/19	714	978	699	270	27.6	73				12.45	79	
8/19	Bled 220 cc.											
8/21	804	1200	858	323	26.9	67	0.84	4,0	26,2	12.35	97	
8/21	Diet: 200 grams crackermeal, 500 cc. milk											
8/27	788	984	667	308	31.3	80	0.80	5,0	12,4	12.50	79	* Poik.
9/4	894	1048	670	367	35.0	85	0.66	6,4	8,6	12.55	84	* Poik. ++
9/11	1078	1135	688	441	38.9	95	0.65	7,3	6,4	12.55	90	* Poik. ++
9/19	1136	1152	700	442	38.4	99	0.67	7,4	18,0	12.60	92	* Poik. ++
9/27	1380	1315	737	558	42.5	105	0.67	7,8	14,8	12.90	102	* Poik. +
10/9	1085	1119	640	497	44.4	97	0.63	7,7	11,0	12.80	93	
10/17	1290	1277	728	536	42.0	101				12.75	100	
10/18	Diet: Mixed diet. Extra meat. Dietary deficiency disease											
10/25	788	1050	690	355	33.8	75	0.58	6,5	10,8	10.35	101	
10/26	Killed. Autopsy											

* Poikilocytosis of red cells.

Refer to table 30, bread and milk experiment.

death 1 week later. Note the fall in blood volume, hemoglobin and red cell hematocrit during this short period. The autopsy findings will not be discussed at this time.

Table 34 presents a long experiment in which the diet is cracker-meal, lard and butter in sufficient amounts to preserve the body weight and permit of a gain of 0.7 kilo during the period of 12 weeks. No previous anemia experiments had been performed on this dog, which

TABLE 34

Blood regeneration—crackermeal, lard and butter. Dog 17-205. Bull mongrel, male, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
10/29	1450	1058	571	487	46	137	0.87	7,9	8,6	10.70	99
10/29	Diet: Bread and milk										
10/30	Bled 265 cc.										
10/31	Bled 265 cc.										
11/2	522	790	563	229	29	66	1.14	2,9	27,0	10.40	76
11/2	Diet: 200 grams crackermeal, 10 grams lard, 10 grams butter										
11/9	559	860	585	275	32	65			6,2	10.30	83
11/16	948	1088	664	425	39	87	1.01	4,3	18,8	10.30	105
11/23	969	1052	589	463	44	92	0.90	5,1	9,2	10.30	102
11/28	998	998	549	449	45	100	0.78	6,4	9,2	10.30	97
12/5	1111	1028	555	473	46	108	0.86	6,3	8,8	10.40	99
12/10	1070	1008	544	464	46	106	0.82	6,5	13,6	10.20	99
12/19	904	913	511	404	44	99	0.77	6,4	13,6	10.60	86
12/26					44	119	0.79	7,5	8,0	10.60	
1/2/18					48	124	0.78	7,9	10,2	10.50	
1/9					46	127	0.79	8,0	12,8	10.70	
1/17					46	127	0.85	7,5	14,2	10.70	
1/23	1480	1139	592	546	48	130	0.69	9,4	13,4	11.10	103

No previous anemia experiments on this dog.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

may account for the fact that the blood regeneration finally carried the level to normal. This reserve has been mentioned in the preceding communication. The gain is very slow and at times there appears to be a slight loss in red cell hematocrit or hemoglobin or pigment volume.

The end result after 12 weeks of this diet may be accepted as normal. It is unusual that this dog tolerated this diet for such a prolonged period without any signs of dietary deficiency disease. A subsequent experiment (paper V) shows dietary deficiency symptoms in this same dog after a shorter period of a similar diet.

TABLE 35

Blood regeneration—crackermeal, lard and butter. Dog 16-160. White bull mongrel, female, age 12 months

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT/TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
3/4	1218	1006	533	473	47	121	0.67	9,1	7,2	10.00	100	
3/4	Diet: Crackermeal, lard and butter											
3/6	Bled 262 cc.											
3/7	Bled 242 cc.											
3/9	726	844	591	256	30	86	0.86	5,0	9,4	9.60	89	
3/9	Diet: 206 grams crackermeal, 10 grams lard, 10 grams butter											
3/15	732	842	581	261	31	87	0.72	6,0	8,4	9.30	91	Slight diarr- rhea
3/20	762	786	517	267	34	97	1.08	4,5	9,8	9.30	84	
3/27	840	785	526	259	33	107	0.89	6,0	8,6	9.20	85	
4/3	812	805	531	274	34	101	0.83	6,1	15,8	8.90	90	
4/9	845	836	535	301	36	101	0.78	6,5	12,6	9.10	92	

Experimental history, see table 18-b.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

The experiment given in table 35 is very much like the preceding one but for the fact that this dog had been observed previously in anemia periods (see experimental history 18-b). The blood regeneration is very slow on the same amount of crackermeal, lard and butter. There was a slight loss of weight on this diet which contains a sufficient number of calories per kilo. This is to be explained by the presence of diarrhea.

The addition of milk powder (table 36) to the lard, butter and crackermeal diet does not modify the curve of blood regeneration nor does it prevent the development of the dietary deficiency disease after a period of 1 month. The peculiar reaction of the red cell hematocrit which actually diminishes as the hemoglobin and red cell count increase may be explained in part by the use of dry oxalate in varying amounts.

TABLE 36

Blood regeneration—crackermeal, lard, butter and milk powder. Dog 16-140. Bull mongrel, male, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
	cc.	cc.	cc.	per cent	per cent					kgm.	cc.	
3/4	2212	1616	711	905	56	137	0.77	8,9	6,8	15.10	107	
3/4	Diet: Crackermeal, lard and butter											
3/6	Bled 414 cc.											
3/7	Bled 394 cc.											
3/9	1027	1222	880	465	38	84	0.87	4,8	13,2	14.30	85	
3/9	Diet: 163 grams crackermeal, 10 grams lard, 10 grams butter, 100 ± grams milk powder											
3/15	1215	1322	846	476	36	92	0.88	5,2	11,2	14.8	89	
3/20	1153	1281	883	448	35	90	0.88	5,1	9,6	15.0	85	
3/27	1055	1227	834	392	32	86	0.84	5,1	8,0	14.80	83	
4/3	1350	1324	834	490	37	102	0.77	6,6	19,8	14.60	91	
4/9	1408	1257	855	402	32	112	0.84	6,7	11,6	14.10	89	*

* Dietary deficiency disease. Death April 17.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

The pigment volume regeneration is about the expected amount in view of the diet which was sufficient to maintain but not increase the body weight.

The next three experiments may be discussed in a group (tables 37, 38 and 39). The influence of splenectomy is concerned in two of these experiments and under these experimental conditions the blood regeneration appears to progress in a normal fashion, at least for a time.

TABLE 36-B
Experimental history. Dog 16-140

EXPERIMENT NUMBER	DIET	BLOOD REGENER- ATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 9/13/16 Bled 450 cc. End 10/13/16	Mixed			kgm. 8.1 8.4 10.1	(3 bleedings)
Begin 11/6/16 Bled 660 cc. End 12/16/16	Fasting, metabolism Sugar, metabolism Gliadin, sugar Gelatin, sugar			9.3 8.2 6.4	(4 bleedings)
Begin 9/11/17 Bled 712 cc. End 10/19/17	Sugar, metabolism Mono-amino-acid fraction of gelatin	1570 756 1273	109 90 130	13.1 12.6 9.3	
Begin 3/4/18 Bled 808 cc. End 4/9/18	Crackermeal, lard, butter, milk pow- der	2210 1025 1385	107 85 89	15.1 14.3 14.1	Table 36 Dietary defi- ciency dis- ease

TABLE 37
Blood regeneration—crackermeal, lard, butter, alfalfa meal. Dog 18-97. Bull
mongrel, female, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
3/11	2038	1358	584	774	57	150	0.88	8,5	9,2	12.40	109
3/11	Diet: Bread and milk										
3/13	Bled 339 cc.										
3/14	Bled 340 cc.										
3/16	908	966	570	396	41	94	1.04	4,5	15,6	11.40	82
3/16	Diet: Crackermeal, lard, butter, 40 grams alfalfa meal										
3/22	1175	1118	548	570	51	105	0.86	6,1	22,2	10.70	103
3/29	1250	1058	561	498	47	118	0.75	7,9	14,6	10.80	98
4/4	1322	1076	581	495	46	123	0.74	8,3	12,4	10.90	99
4/10	1598	1102	595	508	46	145	0.75	9,7	11,4	11.00	100

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.
No previous anemia experiments on this dog.

TABLE 38

*Blood regeneration—crackermeal, lard and butter—splenectomy. Dog 17-163.
Bull mongrel, male, young adult*

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
9/3	1110	1069	588	481	45	104	0.72	7,2	6,2	10.0	106	
9/3	Diet: Bread and milk											
9/4	Bled 279 cc.											
9/5	Bled 279 cc.											
9/7	388	719	532	187	26	54	0.90	3,0	22,4	9.70	74	
9/7	Diet: 200 grams crackermeal, 20 grams lard											
9/14	610	813	512	301	37	75	1.01	3,7	10,2	9.60	85	
9/21	728	867	512	355	41	84		5,8	5,6	9.60	91	
9/22	Diet: 200 grams crackermeal, 10 grams lard, 10 grams butter											
9/28	754	820	492	328	40	92	0.72	6,7	8,0	9.60	85	
10/5	704	800	456	344	43	88	0.73	6,0	13,4	9.80	80	
10/12	806	848	517	331	39	95	0.82	5,8	5,6	9.70	87	
10/17	886	914	539	375	41	97	0.88	5,5	6,6	9.70	94	
10/25	868	914	585	329	36	95	0.79	6,0	8,0	9.80	93	
10/31	145	596	518	78	13	24	0.75	1,6	20,4	9.70	61	*

* Death. (Internal hemorrhages, urobilin in urine ++.)

Blood volume by dry oxalate. Hemoglobin by Sahli tubes.

TABLE 38-B

Experimental history. Dog 17-163 (splenectomy)

EXPERIMENT NUMBER	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 4/24/17	Meat, Blaud's pills	918	102	kgm. 9.0	Table 75
Bled 458 cc.		340	76	8.9	
End 6/6/17		1063	117	8.6	
Begin 9/3/17	Crackermeal, lard Crackermeal, lard, butter	1112	106	10.0	Table 38
Bled 558 cc.		388	74	9.7	
9/21/17		728	91	9.6	
10/25/17		868	93	9.8	Death 11/1/17
End 10/31/17		143	61	9.7	

Table 38 illustrates a not infrequent condition which develops in splenectomized dogs made anemic and fed on a limited diet. We have pointed out elsewhere (1) that there is a remarkable condition which may develop in splenectomized bile fistula dogs. In these bile fistula dogs if anemia is produced we may observe periods of spontaneous blood destruction and enormous pigment overproduction. Under such conditions it was suggested that the body was forming its maxi-

TABLE 39

Blood regeneration—crackermeal, lard, butter, alfalfa meal—splenectomy. Dog 17-34. Bull mongrel, female, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
3/11	1776	1345	740	606	45	132	0.94	7,0	11,8	16.10	84
3/11	Diet: Bread and milk										
3/13	Bled 336 cc.										
3/14	Bled 336 cc.										
3/16	828	1034	765	269	26	80	1.14	3,5	22,0	15.30	68
3/16	Diet: Crackermeal, lard, butter, 50 grams alfalfa meal										
3/22	1128	1128	722	407	36	100	1.00	5,0	17,4	14.80	76
3/29	1282	1187	760	427	36	108	0.93	5,8	17,0	15.20	78
4/4	1185	1162	732	430	37	102	0.77	6,6	16,2	15.00	77
4/10	1371	1193	740	453	38	115	1.08	5,3	16,8	14.80	81

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

Experimental history, see table 17-b.

mum amounts of pigment material (hemoglobin as well as bile pigment) but the red cell stroma was lacking in quantity or quality. It was suggested that the spleen might be concerned in the development of red cell stroma. These present experiments may point to the production of faulty red cell stroma under such conditions (table 38) but some readers may wish to postulate the development of some unknown poison to explain the disintegration of the red cells in our splenectomy experiments. This condition develops very abruptly and may super-

vene in a dog with relatively normal red cell count and hemoglobin values. Within 2 or 3 days the red cell count may fall to one-third normal (table 38) and abnormal pigments appear in blood serum and urine. Much more study must be given to this condition and we expect to report further work in this line.

Alfalfa meal was added to the diets in two of these experiments. These experiments give no evidence to indicate that alfalfa meal exerts a definite influence upon the curve of hemoglobin regeneration in the dog. The alfalfa meal used in our experiments was the usual grade of finely ground alfalfa purchased on the open market.

Rice, potatoes and milk. We may consider the next group of experiments as a unit (tables 40, 41, 42, 43 and 44). In principle all these experiments are similar and the results are remarkably uniform. In the first four experiments the dogs were bled and placed upon a uniform diet of cooked rice, boiled potatoes and skim milk. The regeneration in most of the experiments was slow but uniform with the end result after 5 to 6 weeks about normal or slightly below the normal blood level. After this there followed a short period (7 to 10 days) of mixed diet. Then a second period of anemia and blood regeneration upon the same rice, potato, milk diet was observed. These second periods are replicas of the first regeneration periods on this same diet.

It is clear that a liberal diet of cooked rice and potato with skim milk sufficient to maintain or slightly increase the body weight will give a slow steady gain in blood pigment, red cell hematocrit, red cell count, etc., which will often bring the regeneration curve back to normal or close to normal.

Two experiments are exceptions to the general reaction (tables 40 and 44). Table 40 shows a regeneration which is incomplete and not back to normal in 5 weeks. In fact, during the last month the regeneration is not in evidence and the pigment volume, hemoglobin and red cell hematocrit are stationary. There was a slight loss of weight during this period but the dog was very active and normal in all respects. The second anemia regeneration period shows an identical reaction. Table 44 shows a still more striking difference from the normal average regeneration. This dog refused to eat the amounts of rice and potato and milk given at first. She ate the amounts recorded in table 44, which amount to about 50 per cent that given to the other dogs, or 50 calories per kilo body weight. During 6 weeks there was a loss of 2.5 kilos and the blood regeneration was only slight during this whole period.

TABLE 40

*Blood regeneration—rice, potatoes and milk—repeat experiment. Dog 19-104.
Bull mongrel pup, male*

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm	cc.	
1/30	1367	1067	455	602	56.4	128						
2/6	1394	1078	468	594	55.1	129	0.68	9,5	14,8	8.75	122	
2/6	Diet: Bread and milk											
2/7	Bled 270 cc.											
2/8	Bled 270 cc. No distress											
2/10	363	630	457	170	26.9	58	0.76	3,8	25,8	8.35	75	
2/10	Diet: 363 grams rice, 417 grams potatoes, 500 cc. milk											
2/17	692	765	451	311	40.6	90	0.82	5,5	11,6	8.00	96	
2/26	933	859	451	404	47.0	109	0.75	7,3	8,4	8.00	107	
3/3	807	816	445	366	44.9	99	0.70	7,1	10,2	7.90	103	* Poik. + †
3/10	910	833	419	410	49.2	109	0.80	6,8	10,0	7.85	106	* Poik.
3/19	814	773	402	362	46.9	105	0.67	7,8	9,0	7.85	98	* Poik. + †
3/21	Diet: Mixed diet. Extra food											
3/31	1036	898	433	456	50.8	115	0.69	8,3	12,2	8.65	104	
3/31	Diet: Bread and milk											
4/1	Bled 225 cc.											
4/2	Bled 225 cc. No distress											
4/3	462	688	462	223	32.4	67	0.88	3,8	24,8	8.20	84	
4/3	Diet: 363 grams rice, 417 grams potatoes, 500 cc. milk											
4/11	656	780	469	300	38.4	84	0.70	6,0	11,2	8.20	97	* Poik.
4/18	701	772	435	330	42.7	91	0.56	8,1	8,8	8.05	96	
4/25	760	764	421	339	44.4	98	0.55	8,9	8,0	8.00	95	
5/2	844	816	421	391	47.9	103	0.64	8,1	7,8	7.90	103	
5/9	791	804	431	369	45.9	98	0.58	8,5	5,8	7.65	105	

* Poikilocytosis of red cells.

† Only 300 cc. of milk given.

‡ Gave 300 grams rice and 300 grams potatoes.

No previous anemia experiments on this dog.

TABLE 41

Blood regeneration—rice, potatoes and milk—repeat experiment. Dog 19-95. Bull mongrel, male, age 5 months

DATE, 1919	PIGMENT VOLUME Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/16	1232	1130	595	528	46.4	109	0.67	8,2	18,0	11.35	100	
1/16	Diet: Crackermeal and milk											
1/17	Bled 282 cc.											
1/18	Bled 282 cc. No distress											
1/20	560	824	602	218	26.4	68	0.77	4,4	30,4	11.00	75	
1/20	Diet: 418 grams boiled rice, 490 grams potatoes, 500 cc. milk											
1/27	742	974	574	395	40.6	76	0.72	5,3	16,2	12.75	76	
2/3	954	976	531	435	44.6	98	0.65	7,5	22,8	11.15	87	
2/12	982	1013	538	464	45.8	97	0.75	6,5	14,2	10.90	93	
2/19	1040	1000	511	480	48.0	104	0.70	7,4	10,6	10.60	95	
2/28	1242	1058	514	538	50.9	118	0.88	6,7	9,8	10.80	98	* Poik. +
3/7	983	1006	521	475	47.2	98	0.87	7,2	10,0	11.20	90	* Poik. +
3/10	Diet: Mixed diet											
3/17	1092	1130	640	484	42.8	97	0.84	5,8	13,0	12.80	88	* Poik.
3/17	Diet: Crackermeal and milk											
3/18	Bled 283 cc.											
3/19	Bled 283 cc. No distress											
3/21	570	934	664	251	26.9	61	0.92	3,3	18,0	12.45	75	* Poik.
3/21	Diet: 418 grams boiled rice, 490 grams potatoes, 500 cc. milk											
3/28	577	834	576	250	30.0	69	0.86	4,0	6,0	11.75	79	* Poik.
4/2	741	953	605	334	35.0	78	0.63	6,2	11,6	11.80	81	* Poik. +
4/9	773	976	600	371	38.0	79	0.70	5,6	8,2	11.85	82	
4/16	988	1046	588	448	42.8	94	0.69	6,8	10,4	11.90	98	
4/23	1085	1073	576	492	45.8	101	0.68	7,4	20,0	11.95	90	
4/30	1237	1168	576	581	49.7	106	0.65	8,1	8,2	11.95	98	

* Poikilocytosis of red cells.

No previous anemia experiments on this dog.

TABLE 42

Blood regeneration—rice, potatoes and milk—repeat experiment. Dog 19-93. Bull mongrel, female, age 5 months

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/16	1435	1380	742	616	44.7	104	0.59	8,9	18,6	10.95	126	
1/16	Diet: Bread and milk											
1/17	Bled 345 cc.											
1/18	Bled 245 cc. No distress											
1/20	594	886	640	237	26.8	67	0.76	4,4	15,2	10.25	86	
1/20	Diet: 400 grams boiled rice, 475 grams potatoes, 500 cc. milk											
1/27	768	1084	638	436	40.2	71	0.48	7,4	10,8	10.35	105	* Slight
2/3	1058	1080	570	494	45.7	98	0.60	8,1	15,2	9.55	113	* Slight Cells small
2/12	1040	1063	566	482	45.3	98	0.69	7,1	8.4	9.90	107	*
2/19	1245	1163	570	582	50.0	107	0.73	7,3	6,4	9.55	122	* Slight
2/28	1255	1125	568	540	48.0	112	0.84	6.7	5,8	9.65	116	* Slight
3/7	1098	1127	568	542	48.1	98	0.73	6,7	6,0	10.35	113	* Slight
3/10	Diet: Mixed diet											
3/17	1040	1004	516	478	47.6	104	0.70	7,4	7,8	11.35	89	* Slight
3/17	Diet: Bread and milk											
3/18	Bled 251 cc.											
3/19	Bled 251 cc. No distress											
3/21	566	916	647	260	28.4	62	0.84	3,7	12,2	11.15	82	*
3/21	Diet: 400 grams boiled rice, 475 grams potatoes, 500 cc. milk											
3/28	896	1028	614	397	38.6	87	0.85	5,1	7,8	11.35	90	
4/2	908	1085	631	433	39.9	84	0.65	6,5	8,6	11.20	97	* Slight
4/9	858	990	568	412	41.6	87	0.60	7,2	6,0	11.35	87	
4/16	1136	1112	565	530	47.7	102	0.67	7,6	8,8	11.30	98	* Slight
4/23	1212	1142	568	557	48.8	106	0.57	9,3	6,8	11.35	100	
4/30	1313	1198	572	604	50.4	110	0.58	9,5	7,4	11.15	107	* Slight

* Poikilocytosis of red cells.

No previous anemia experiments on this dog.

TABLE 43

*Blood regeneration—rice, potatoes and milk—repeat experiment. Dog 19-103.
Bull mongrel, female, age 5 months*

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
1/30	1100	1077	588	483	44.9	102				9.55	113	
2/6	978	1020	598	428	41.9	96	0.61	7,9	12,6	10.00	102	
2/6	Diet: Bread and milk											
2/7	Bled 255 cc.											
2/8	Bled 255 cc. No distress											
2/10	430	808	600	204	25.3	53	0.78	3,4	9,0	9.45	85	
2/10	Diet: 411 grams rice, 472 grams potatoes, 500 cc. milk											
2/17	770	854	485	365	42.7	90	0.78	5,8	12,4	8.85	96	
2/26	996	981	520	446	45.5	102	0.68	7,5	10,8	9.10	108	
3/3	1305	1070	491	574	53.6	122	0.80	7,6	8,4	9.20	116	
3/10	1068	1008	526	476	47.3	106	0.68	7,8	7,0	9.20	110	
3/19	1065	986	499	477	48.4	108	0.72	7,5	9,0	9.60	103	*
3/21	Diet: Mixed diet. Extra food											
3/31	1103	1103	600	492	44.6	100	0.71	7,0	10,6	10.65	103	
3/31	Diet: Bread and milk											
4/1	Bled 276 cc.											
4/2	Bled 276 cc. No distress											
4/3	444	837	612	220	26.3	53	0.76	3,5	11,6	10.40	80	
4/3	Diet: 411 grams rice, 472 grams potatoes, 500 cc. milk											
4/11	756	964	603	347	36.0	79	0.76	5,2	8,8	10.70	90	
4/18	803	957	570	377	39.4	84	0.57	7,4	14,6	10.55	91	
4/25	970	1003	546	439	43.8	97	0.64	7,6	7,2	10.65	94	
5/2	1077	1057	560	491	46.5	102	0.62	8,2	11,0	10.35	102	
5/9	1045	1062	592	460	43.3	98	0.65	7,5	14,6	10.50	101	

* Poikilocytosis of red cells.

No previous anemia experiments on this dog.

This experiment (table 44) shows admirably a reaction noted in other experiments. The bleeding reduced the hemoglobin from 123 per cent to 59 per cent and the red cells from 7,500,000 to 3,300,000. During the 6 weeks' observation we note a rise of only 24 per cent hemoglobin but the red count returns to normal. The color index of course

TABLE 44

Blood regeneration—rice, potatoes and milk. Dog 19-96. Bull mongrel, female, age 8 months

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
3/17	1520	1238	568	663	53.6	123	0.82	7,5	11,4	13.50	92	
3/17	Diet: Crackermeal and milk											
3/18	Bled 310 cc.											
3/19	Bled 310 cc. No distress											
3/21	527	891	636	246	27.6	59	0.89	3,3	14,2	12.40	72	
3/21	Diet:† 200 grams boiled rice, 200 grams potatoes, 500 cc. milk											
3/28	616	835	551	267	32.0	74	0.93	4,0	14,8	11.55	79	
4/2	662	911	598	308	33.4	73	0.65	5,6	11,4	11.20	81	* Poik.++
4/9	784	959	581	368	38.4	82	0.59	6,9	10,4	10.75	89	* Poik.+
4/16	728	908	550	354	39.0	80	0.56	7,1	6,8	10.30	88	* Poik.+
4/23	818	905	532	368	40.7	90	0.61	7,4	10,2	10.10	90	* Poik.++
4/30	778	938	535	394	42.0	83	0.58	7,2	12,0	9.90	95	* Poik.++

* Poikilocytosis of red cells.

† Animal refused to eat larger quantities of food. Represents about 50 calories per kilo of body weight.

Experimental history, see table 4-b.

drops from 0.93 to 0.58 and poikilocytosis is very much in evidence. Under such conditions one feels a very strong probability of red cell fragmentation.

This evidence (tables 40 and 44) confirms our belief that the *amount of any diet* may be a considerable factor in blood regeneration. Given a diet of a limited nature but sufficient to permit of slight gain in body

weight and we may expect a certain amount of blood regeneration, at times even a return to normal. But given a limited diet in small amounts not sufficient for maintenance of body weight, we may confidently expect a very slow blood regeneration or complete absence of active regeneration. Under such circumstances the body may even be unable to make up its blood cell maintenance factor and the pigment volume curve may actually fall. This is a favorable time for

TABLE 45

Blood regeneration—casein, sugar, butter and lard. Dog 18-56. Bull mongrel, female, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
10/29	1275	930	400	530	57	137	0.89	7,7	12,6	8.40	111
10/29	Diet: Bread and milk										
10/30	Bled 233 cc.										
10/31	Bled 233 cc.										
11/2	340	548	400	148	27	62	1.20	2,6	16,0	7.50	73
11/2	Diet: 75 grams casein, 25 grams sugar, 20 grams butter, 20 grams lard										
11/9	428	586	375	211	36	73	1.30	2,8	19,6	7.50	73
11/16	786	827	463	364	44	95	0.99	4,8	6,0	8.10	102
11/23	725	763	412	351	46	95	0.93	5,1	12,0	7.20	106
11/28	908	810	389	421	52	112	0.81	6,9	12,2	7.30	111
12/5	1330	985	384	601	61	135	0.85	7,9	13,2	7.40	133

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

No previous anemia experiments on this dog.

the appearance of a characteristic dietary deficiency disease which is much like scurvy in human beings and is rapidly fatal if not energetically treated with antiscorbutic measures.

Casein and gliadin. When we consider a bread and milk diet from the standpoint of dietary factors we are obviously dealing with many known and unknown constituents. Two of the familiar ingredients in this bread and milk diet are casein and gliadin, which are concerned

particularly in the following group of experiments (tables 45 to 47 inclusive). Casein used in these experiments was obtained from a large dairy products company in this state. It appears as a fine dry granular powder, pale yellow in color, and is of reasonable purity, judging from information given us by the chemist of this company.

The gliadin was extracted from wheat flour in this laboratory by use of dilute alcohol (70 per cent). The weighed amount of gliadin was thoroughly mixed with the sugar, moistened with water and fed to the dog by spoon. Total ingestion was readily accomplished in this way.

Table 45 shows the influence of casein, sugar, lard and butter on blood regeneration. The diet was sufficient to maintain body weight and the blood regeneration was complete in 5 weeks. We must not forget that this dog had not been used for anemia experiments previous to this time and such dogs occasionally show remarkable regenerative capacity on limited diets. The next experiment, however, is conclusive and shows the effect of casein under more carefully controlled and less favorable conditions.

The second casein experiment (table 46) is preceded by a 2 weeks' sugar diet period during which the expected reaction is noted. There is the usual gain in red cell hematocrit, hemoglobin and pigment volume. If the sugar diet had been continued we are reasonably certain that the pigment volume would have remained stationary or even have fallen. Casein added to the diet shows a distinct gain which is held during the subsequent weeks when we see slight fluctuations in pigment volume but relatively little change. There is a slight gain in weight as the calories in the diet are increased by the use of fats. The figures given for the urinary nitrogen show the normal level for the sugar periods and during the sugar and casein intervals indicate the amount of nitrogenous metabolism.

Table 47 is to be compared with a preceding experiment (table 21, paper II). In both experiments a gliadin sugar diet is used over a period of several weeks. This experiment shows less conclusive evidence of the influence of gliadin upon blood regeneration. We may conclude that the gliadin in this experiment (table 47) was without influence on the curve of blood regeneration. We are inclined to the opinion, however, that sugar alone over this period in this experiment would have been associated with a definite loss in pigment volume by the end of the 6-week period. This experiment shows a transient increase in pigment volume which is lost during the last 2 weeks.

TABLE 46

Blood regeneration—casein, sugar, lard and butter. Dog 16-158. Coach mongrel, male, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	AVERAGE DAILY URIN- ARY NITROGEN	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	grams	
10/17	1817	1367	588	779	57	133	0.74	8,9	11,0	9.70	141		
10/17	Diet: Bread and milk												
10/19	Bled 342 cc.												
10/21	Bled 342 cc.												
10/22	576	823	576	247	30	70	0.95	3,7	11,2	9.50	87		
10/22	Diet: 75 grams cane sugar, 25 grams glucose, 300 cc. water												
10/29	707	895	573	312	36	79	0.79	5,0	10,6	8.60	104	1.81	
11/5	766	958	575	383	40	80	0.67	6,0	6,0	7.90	121	1.61	
11/5	Diet: 100 grams sugar, 50 grams casein, 300 cc. water												
11/12	856	961	567	394	41	89	0.73	6,1	3,8	7.80	123	4.70	
11/19	1038	1081	616	465	43	96	0.67	7,2	5,2	7.70	140	6.60	Vomiting
11/21	Diet: 100 grams sugar, 100 grams casein, 300 cc. water												
11/26	878	944	538	406	43	93	0.69	6,7	5,0	7.80	121	9.36	
12/3	824	970	582	388	40	85	0.75	5,7	6,2	8.00	121	11.60	
12/3	Diet: 100 grams sugar, 100 grams casein, 10 grams lard, 10 grams butter												
12/10	876	974	604	370	38	90	0.57	7,9	9,8	8.30	117		
12/19	876	952	600	352	37	92	0.69	6,7	14,0	8.70	108		
12/20	Diet: 100 grams sugar, 125 grams casein, 10 grams lard, 10 grams butter												
12/26					40	96	0.61	7,8	9,0	8.90			
1/2/18					38	93	0.55	8,4	7,8	9.10			
1/9					35	92	0.62	7,4	8,6	9.30			
1/17					38	85	0.50	8,5	6,8	9.10			
1/23	975	975	614	361	37	100	0.58	8,7	6,2	8.90	109		

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

TABLE 46-B

Experimental history. Dog 16-158

EXPERIMENT NUMBER	DIET	BLOOD REGENER- ATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 9/26/16 Bled 600 cc End 11/20/16	Meat			kgm. 8.1 7.7 9.5	(4 bleedings) Complete regen- eration of Hb. and R.B.C.
Begin 2/12/17 Bled 726 cc. End 4/11/17	Beef heart	1523 385 1476	147 85 152	9.9 9.8 8.8	Table 56
Begin 5/7/17 Bled 660 cc. End 6/18/17	Sugar, gliadin Metabolism	1505 458 456	146 84 113	9.0 8.4 6.2	Table 47 R.B.C. fragment- ed, shadow forms
Begin 10/17/17 Bled 684 cc. End 12/3/17	Sugar Sugar and casein Metabolism	1818 576 825	141 87 121	9.7 9.5 8.0	Table 46
Begin 5/3/18 Bled 698 cc. End 6/18/18	Sugar, glycocoll Metabolism	1800 528 852	119 76 99	11.7 10.9 8.05	Maximum regen- eration 3 weeks, then drop
Begin 8/28/18 Bled 929 cc. End 9/30/18	Sugar and gela- tin	974 548 508	82 88 90	10.3 9.15 7.45	(5 bleedings) Maximum regen- eration 2 weeks
Begin 2/20/19 Bled 934 cc. End 3/18/19	Sugar and liver residue	1862 478 738	111 70 82	12.35 11.10 9.15	Table 67 (3 bleedings)
Begin 8/18/19 Bled 851 cc. End 10/24/19	Beet tops Spinach	1468 560 785	93 77 86	12.55 12.10 10.50	(3 bleedings)

These experiments are not conclusive but give evidence that moderate amounts of gliadin with sugar alone do not modify profoundly the blood regeneration curve. That gliadin in combination with other factors may have a more favorable influence on red cell production may be granted. Also under the conditions of the experiment it appears that casein has a more favorable influence on blood regeneration than gliadin when given with sugar only.

TABLE 47

Blood regeneration—sugar and gliadin. Dog 16-158. Coach mongrel, male, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
5/7	1505	1320	581	740	56	114	0.57	10,1	6,6	9.00	146	
5/8	Bled 330 cc.											
5/9	Bled 330 cc.											
5/11	458	705	480	226	32	65	0.64	5,1	10,8	8.40	84	
5/11	Diet: 50 grams cane sugar, 25 grams dextrose, 25 grams gliadin, 300 cc. water											
5/14	434	804	547	257	32	54	0.68	4,0	9,0	8.20	98	
5/21	435	791	538	253	32	55	0.59	4,7	8,6	7.80	101	*
5/28	596	961	519	346	36	62	0.56	5,5	9,8	7.40	129	
6/4	604	863	552	311	36	70	0.61	5,7	8,4	6.90	122	
6/11	559	766	528	237	31	73	0.61	6,0	9,2	6.60	111	*
6/18	456	702	477	225	32	65	0.53	6,1	11,6	6.20	113	*

* Fragmentation of red blood cells.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

Experimental history, see table 46-b.

SUMMARY

A diet of dried white bread and skim milk may cause a slow, steady gain in blood pigment volume from week to week. A liberal diet of this type sufficient to maintain or increase body weight will often suffice for complete blood regeneration. A restricted diet of bread and

milk barely sufficient for body maintenance will rarely permit of complete blood regeneration following simple secondary anemia.

Repeat experiments done after short intervals of rest to permit complete return to normal condition will show identical reactions on the part of the hemoglobin, red cells and pigment volume. The animal shows no increased ability to produce hemoglobin and red cells after repeated experiments nor is there any evidence for a failure of red cell production under these conditions.

Bile fistula dogs presenting complete exclusion of bile pigments from the intestine show a reaction which is practically identical with that of normal dogs.

Crackermeal (a mixture of wheat flour, barley flour and rice flour) with milk or lard and butter, gives a blood pigment reaction following anemia which is similar to the familiar bread and milk reaction.

A dietary deficiency disease may develop in these dogs kept on limited diets for many weeks. This condition clinically resembles scurvy in human beings and may be prevented or cured by antiscorbutic measures. This question will be reviewed in a subsequent publication.

Splenectomy may not modify the expected reaction of red blood cells following anemia. In certain splenectomy experiments there develops a peculiar condition associated with spontaneous destruction or disintegration of circulating red cells. This may appear following a limited diet of several weeks and runs a very rapid course resulting in death within a few days.

Rice, potatoes and skim milk make up a diet which may be classed with bread and milk as regards its influence upon red blood cell regeneration following the unit hemorrhages. If anything, this diet is slightly more efficient than bread and milk in promoting blood regeneration.

Casein and gliadin by themselves are not efficient factors in promoting red cell regeneration but casein appears to be the more efficient in the amounts used and under the conditions of these experiments.

Any one of these diet mixtures in proper amounts may be used to maintain the pigment volume at a constant level following the initial 2 weeks' blood reaction. Under such conditions any added food factor may be measured with some accuracy as to its power of aiding in blood regeneration.

BIBLIOGRAPHY

- (1) HOOPER AND WHIPPLE: This Journal, 1917, xliii, 275.

BLOOD REGENERATION FOLLOWING SIMPLE ANEMIA

IV. INFLUENCE OF MEAT, LIVER AND VARIOUS EXTRACTIVES, ALONE OR COMBINED WITH STANDARD DIETS

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Cooked meat and liver stand in striking contrast to the milk, bread, potato mixtures outlined in the preceding paper of this series. Cooked liver, lean beef or beef heart alone or in combination are very efficient in bringing about a rapid blood regeneration following the standard type of secondary anemia. These substances are very similar but for the present we may say their efficiency is in the order given; that is, cooked liver is most effective in anemia provided a sufficient amount (caloric value) is eaten, and cooked beef heart is least effective; but the differences are not great and this order may be changed with the accumulation of more data.

These three substances are efficient in stimulating blood regeneration whether given alone or in combination, or together with carbohydrate or mixed diets. They all stand the severe test of promoting definite blood regeneration when administered after long limited diet periods unfavorable to blood regeneration.

Meat extract (commercial) has no value in the blood regeneration complex. But a watery liver extract seems to exert a distinct influence on the blood regeneration. Liver residue (after the watery and alcoholic extraction) alone exerts a definite influence on blood regeneration. We do not wish to go into a discussion of this question of tissue extracts until we present much more experimental data dealing with this and other material of similar nature.

EXPERIMENTAL OBSERVATIONS

In general the experimental technique has been detailed in the first paper of this series. All meat and liver were cooked thoroughly in boiling water before feeding, with the exception of "meat scraps."

The meat scraps were obtained from the University Hospital and included meats of various kinds cooked in different ways. A certain amount of fat was of necessity included in this meat diet. Unless otherwise noted these diets were completely ingested. With an occasional exception noted in the tables the dogs were in uniformly excellent condition.

TABLE 48

Blood regeneration—cooked meat scraps. Dog 17-38. Bull mongrel, female, adult

DATE, 1920	PIGMENT VOLUME Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
2/12	1322	1268	670	591	46.6	104	0.71	7,3	7,4	10.90	115
2/12	Diet: Bread and milk										
2/13	Bled 317 cc.										
2/14	Bled 317 cc.										
2/16	550	1000	714	281	28.1	55	0.75	3,7	6,2	10.35	97
2/16	Diet: 500 grams cooked meat scraps										
2/24	948	1168	713	435	37.2	80	0.69	5,8	10,8	11.05	106
3/1	1046	1236	742	485	39.2	85	0.66	6,4	5,8	11.50	107
3/8	1437	1332	682	636	47.8	108	0.68	7,9	9,8	12.10	110
3/15	1332	1378	734	626	45.4	97	0.62	7,8	7,4	12.85	107
3/22	1475	1420	704	704	49.5	104	0.62	8,4	8,2	13.20	108

Experimental history, see table 20-b.

The first two tables show the characteristic reaction to a diet of meat scraps. There is a prompt and rapid regeneration of hemoglobin and red cells which brings the hematocrit and pigment volume back to practically normal in 3 weeks. This level is sustained for the subsequent 2 weeks. The first experiment (table 48) shows a rather low initial level (104 per cent hemoglobin) but a very prompt reaction following the anemia. The hemoglobin, red cell hematocrit and pigment volume return to a level slightly above the original normal level. There was a marked gain in weight.

The second experiment (table 49) required a third bleeding to reduce the red cells to the usual anemia level. The regeneration of the red cells is practically complete in 3 weeks, although the previous high level of hemoglobin and red cell hematocrit is not reached. This level is uniform during the next 2 weeks. In both experiments the plasma volume is relatively constant during the entire period of observation.

TABLE 49

Blood regeneration—cooked meat scraps. Dog 18-114. Bull mongrel, female, adult

DATE, 1920	PIGMENT VOLUME = Hb. PERCENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
2/12	1995	1705	766	931	54.6	117	0.70	8,4	10,8	15.50	110
2/12	Diet: Bread and milk										
2/13	Bled 425 cc.										
2/14	Bled 425 cc.										
2/16	822	1232	818	403	32.7	67	0.98	3,6	7,8	14.30	86
2/16	Bled 275 cc.										
2/18	Diet: 600 grams cooked meat scraps										
2/24	1135	1480	950	504	34.0	77	0.80	4,8	10,2	14.25	104
3/1	1434	1525	857	640	42.0	94	0.73	6,4	9,0	14.35	106
3/8	1580	1540	798	726	47.2	103	0.68	7,6	8,2	15.15	102
3/15	1678	1678	874	796	47.4	100	0.63	7,9	7,8	15.50	108
3/22	1787	1728	838	864	50.0	103	0.63	8,2	9,4	15.50	111

Experimental history, see table 12-b.

Table 50 presents a reaction very much like that noted in the two preceding experiments. Fresh lean beef in adequate amounts was fed. It was cooked in boiling water. This meat contained very little fat and was purchased under the trade name of "chuck." The dog shows a very high level before bleeding and three bleedings did not reduce the level to the usual anemia level. This dog had not been used previously in anemia experiments. The blood regeneration was rapid in the first

2 weeks, but there was little gain in the third week except in the red count, which shows a remarkable jump. This gives a corresponding fall in the color index. A change to a mixed diet shows but little change until the third week of mixed diet when the original high level was attained.

TABLE 50

Blood regeneration—lean beef. Dog 18-24. Bull mongrel, male, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
11/14	1560	1300	480	814	62.6	120	0.87	8,4	12,0	13.20	99
11/12	Diet: Crackermeal and milk										
11/15	Bled 325 cc. Slight distress										
11/16	Bled 195 cc.										
11/18	582	915	612	298	32.6	64				12.70	72
11/18	Bled 150 cc.										
11/20	466	806	539	264	32.7	58	0.81	3,6	20,2	11.85	68
11/20	Diet: 567 grams cooked lean beef										
11/27	935	995	590	399	40.1	94	0.80	5,9	15,8	12.70	78
12/4	1093	1062	585	473	44.5	103	0.61	8,4	14,9	13.15	81
12/11	1090	1033	582	466	45.1	106	0.48	11,9	15,0	13.40	77
12/13	Diet: Mixed diet										
12/20	1055	1115	594	511	45.8	95	0.47	10,1	25,8	13.50	83
12/27	1010	1060	544	516	48.6	95				13.20	80
1/10/19	1425	1230	550	674	54.8	116	0.58	10,1	7,8	13.20	93

No previous anemia experiments on this dog.

Table 51 illustrates a reaction which is in no sense typical, but complicated by abnormal factors. It is submitted with reservations. This dog has been used in a variety of blood regeneration experiments (table 6-b), therefore the type of reaction is well established. But within a few weeks following the present experiment the dog died with bilateral

TABLE 51

Blood regeneration—lean beef and gelatin—lean beef and brain. Dog 17-28. Bull mongrel, female, young adult

DATE, 1918-1919	PIGMENT VOLUME = Hb. PERCENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
	cc.	cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
12/2	2118	1650	740	903	54.7	128	0.58	11,0	9,8	15.70	105	
12/2	Diet: Crackermeal and milk											
12/3	Bled 413 cc.											
12/4	Bled 413 cc.											
12/6	1000	1256	850	394	31.3	79				15.30	82	
12/7	Bled 314 cc.											
12/9	708	1240	926	300	24.2	57	0.79	3,6	15,6	15.05	82	
12/9	Diet: 681 grams cooked lean beef, 20 grams cooked gelatin—100 calories per kilo											
12/16	916	1063	636	388	36.5	86	0.84	5,1	26,2	13.35	80	See foot-note
12/17	Diet: 100 grams cooked brain, 580 grams lean beef—100 calories per kilo											
12/23	954	1047	607	412	39.4	91	0.53	8,5	19,0	12.90	81	See foot-note
12/23	Diet: Mixed diet											
12/30	883	1100	722	366	33.3	80				13.35	82	* Poik. ++ *
1/8/19	1162	1263	720	531	42.0	92	0.69	6,7	10,2	13.50	94	
1/15	1050	1290	750	530	41.0	82	0.64	6,4	10,6	14.05	92	
1/22	1380	1450	792	635	43.8	95	0.64	7,4	17,4	14.65	99	

* Poikilocytosis of red blood cells.

December 16, 1918: Food not touched. Seems sick. Drank considerable water this a.m. and immediately afterward vomited it. Temperature, 38.4°C. Abdomen seems distended. Gave 150 grams meat and 50 grams crackermeal.

December 23, 1918: Left 300 grams food. Very thirsty, vomits water within 5 minutes after drinking. Temperature, 38.8°C. Inactive, slight dragging of right hind leg. Put in metabolism cage; 400 cc. water; mixed diet.

December 26, 1918: Animal recovered. Is still thirsty.

Subsequent death. Stone in kidney.

Experimental history, see table 6-b; see autopsy, table 14.

renal calculi. It is therefore certain that this dog was suffering from renal disease during this experiment (table 51). We note in this experiment that the dog lost weight rapidly on the diet of beef and gelatin which was not eaten with relish. Finally the diet was changed as

TABLE 52

Blood regeneration—beef heart and liver. Dog 18-116. Bull mongrel, female, young adult

DATE, 1918-1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
12/2/18	2120	1720	805	907	52.7	123	0.56	11.4	14.1	14.90	115
12/2	Diet: Crackermeal and milk										
12/3	Bled 430 cc.										
12/4	Bled 430 cc.										
12/6	939	1183	803	369	31.2	79	0.57	6.9	21.2	14.55	81
12/7	Bled 300 cc.										
12/9	776	1260	930	323	25.6	62	0.84	3.7	17.5	13.95	90
12/9	Diet: 256 grams cooked beef heart,* 610 grams cooked beef liver*—100 calories per kilo										
12/16	1082	1330	844	476	35.8	81	0.88	4.6	18.2	14.55	91
12/23	1890	1685	860	818	48.5	112	0.57	9.9	11.5	15.25	110
12/30	2270	1648	747	902	54.7	138				15.50	106
12/30	Diet: Mixed diet										
1/8/19	2040	1715	785	912	53.2	119	0.72	8.3	7.0	15.50	110
1/15	1760	1610	762	842	52.2	109	0.68	8.0	12.0	16.20	99
1/22	2120	1740	773	948	54.4	122	0.71	8.6	10.6	15.65	111

* Meat cooked, fat and connective tissue removed, and ground.

Experimental history, see table 13-b.

the beef and gelatin mixture was refused. The beef and brain mixture was also eaten poorly, and more weight was lost. We are fortunate in being able to refer to a fasting experiment on this same dog (table 14) which shows practically an identical gain during two weeks as recorded

in table 51. The distaste for the food mixture which was eaten only in part we believe is largely responsible for this lack of blood regeneration. We see that the renal disease does not modify the expected blood reaction during a fasting period (table 14) and we have no right to assume that it might seriously modify the reaction in table 51.

TABLE 53

Blood regeneration—beef heart. Dog 19-6. Bull mongrel, male, young adult

DATE, 1918-1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
11/14	2082	1543	590	946	61.3	135	0.77	8,8	11,0	14.00	110
11/14	Diet: Crackermeal and milk										
11/15	Bled 386 cc.										
11/16	Bled 386 cc. No distress										
11/18	800	1020	650	365	35.8	78				13.00	79
11/18	Bled 200 cc.										
11/20	697	1026	710	317	30.8	68	0.74	4,6	16,4	12.80	80
11/20	Diet: 431 grams cooked beef heart*—100 calories per kilo										
11/27	1325	1250	670	574	45.9	106	0.79	6,7	6,0	12.55	100
12/4	1480	1170	568	601	51.1	126	0.64	9,8	6,7	12.75	92
12/11	1642	1325	637	681	51.4	124	0.62	10,5	10,2	12.25	108
12/13	Diet: Mixed diet										
12/20	1376	1188	588	588	49.5	116	0.58	10,0	11,4	12.50	95
12/27	1287	1226	642	579	47.2	105				13.15	93
1/10/19	1940	1462	607	840	57.5	133	0.81	8,2	7,0	13.20	110

* Beef heart cooked, fat and connective tissue removed, and ground.

No previous anemia experiments on this dog.

Table 52 illustrates an optimum reaction on a diet of beef heart and beef liver. Both these substances favor a rapid blood regeneration especially when a sufficient amount is eaten. This dog ate the mixture with relish and gained over 1 kilo during 3 weeks. There is a truly

remarkable gain in red cells, hemoglobin and pigment volume. During 3 weeks the regeneration is complete and the anemia level (3 bleedings) if anything was below the average level. Many other anemia experiments have been completed on this dog (table 13-b) and the type

TABLE 54

Blood regeneration—beef heart. Dog 19-84. Bull mongrel pup, female

DATE, 1918-1919	PIGMENT VOLUME = Hb. PERCENT TIMES BLOOD VOLUME			BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT		Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
	cc.	cc.	cc.	cc.	per cent	per cent							kgm.	cc.	
12/2	992	780	352	424	54.4	127	0.53	12,0	10,6	6.95	112	R. B. C. small			
12/2	Diet: Crackermeal and milk														
12/3	Bled 195 cc. No distress														
12/4	Bled 125 cc. No distress														
12/6	448	491	305	183	37.3	91	0.68	6,7	24,0	6.10	80				
12/7	Bled 123 cc.														
12/9	428	542	360	177	32.6	79	1.20	3,3	17,1	5.85	93				
12/9	Diet: 228 grams beef heart*—100 calories per kilo														
12/16	638	608	332	274	45.0	105	0.86	6,1	10,1	5.80	105	R. B. C. small			
12/23	796	676	328	345	51.0	118	0.59	10,8	15,2	5.90	115				
12/30	968	711	323	387	54.5	136				5.90	120				
12/30	Diet: Mixed diet														
1/8/19	855	750	366	380	50.5	114	0.71	8,0	23,8	6.95	107				
1/15	712	818	405	409	50.0	87	0.61	7,1	10,8	7.80	105				
1/22	1020	937	476	456	48.7	109	0.55	10,5	14,2	7.50	125				
1/30	987	888	429	455	51.2	111	0.75	7,4	11,2	7.50	118				

* Beef heart cooked, fat and connective tissue removed, ground.

No previous anemia experiments on this dog.

reaction is therefore established. It may be stated even that the regeneration was *almost complete within 2 weeks*.

With the change to mixed diet we note a reaction which is not uncommon when a sudden change is made from a fixed diet to another

very different diet. Under such conditions even when the second diet is most favorable we may record a slight fall in red cell hematocrit, hemoglobin and pigment volume. This experiment, too, shows a fall in the red count. We have no good explanation to offer, but this fact is to be considered in the proper interpretation of various tables.

TABLE 55

*Blood regeneration—beef heart and lean meat following 3 weeks of sugar—metabolism.
Dog 16-157. Bull mongrel, male, age 10 months*

DATE, 1916-1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
12/14/16						116		7,9	6,4	8.10		
12/14	Diet: Sugar (3 weeks)											
1/5/17	546	976	625	351	36	56	0.55	5,1	7,2	6.10	160	
1/5	Diet: 400 grams beef heart, 100 grams sugar											
1/11					43	76	0.58	6,6	11,8	6.60		
1/13	Diet: Lean meat											
1/17	408	908	563	345	38	45	0.44	5,1	6,4	7.60	119	*
1/26	738	838	478	360	43	88	0.59	7,5	8,2	8.00	105	*
2/2	838	998	529	469	47	84	0.57	7,3	7,0	8.20	122	
2/16	1130	1202	553	649	54	94	0.53	8,8	9,8	9.20	131	
2/23	1052	1052	526	526	50	100	0.58	8,6	13,4	9.20	115	
3/2	1208	1220	549	672	55	99	0.56	8,9	9,8	9.20	132	

* Anisocytosis and poikilocytosis of red blood cells.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

Cooked beef heart is a food commonly used in experimental laboratories. It is usually assumed that heart muscle as a food is very like skeletal muscle, although there may be certain differences as pointed out by Mendel and Osborne (1). We have found that it compares favorably with skeletal muscle as far as concerns the regeneration of red cells and hemoglobin. Whether beef heart is actually identical with lean beef in its effect on blood regeneration cannot be stated posi-

tively as we cannot at this time submit a sufficiently complete series of controlled experiments.

Tables 53 and 54 are identical in all essential factors. Both dogs were used for the first time and we must consider the unusual "reserve" which at times may be demonstrated by such dogs. The cooked beef heart was eagerly eaten, and the weight was practically stationary, although the first dog lost a little during the third week. Blood regeneration was practically complete in 3 weeks as regards red cells,

TABLE 55-B

Experimental history. Dog 16-157

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 9/26/16 Bled 600 cc. End 10/11/16	Fasting			<i>kgm.</i> 7.90 6.80 5.40	(4 bleedings) Slight regeneration of Hb. and R. B. C.
Begin 12/14/16 Bled 600 cc. End 3/2/17	Sugar, metabolism Sugar, beef heart Beef heart	1208	132	8.10 7.30 9.20	(4 bleedings)
Begin 5/7/17 Bled 648 cc. End 6/11/17	Gelatin, sugar Metabolism	1570 429 792	130 74 126	10.00 9.20 6.30	
6/14	Killed—bled from carotid				

hemoglobin and pigment volume. Both dogs showed a distinct drop when changed to a mixed diet,—a change recorded in red cell hematocrit, hemoglobin and red count. There was no change in plasma volume to explain this fluctuation and for the present we must be content with recording this fact without advancing any convincing explanation (refer to exper. 52). A similar fall is noted in table 66 even when the change is from a poor diet (bread and milk) to a more favorable diet (beef heart).

Table 55 illustrates the reaction on beef heart diet following a 3-week period of sugar feeding. After the meat diet is established we note a slow but steady gain back to normal in 4 to 5 weeks. We wish to emphasize the fact that a period of fasting or sugar feeding makes subsequent blood regeneration much more difficult and this is a severe test for any food substance. Only meat (including beef heart) and

TABLE 56

Blood regeneration—beef heart. Dog 16-158. Coach mongrel, male, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
2/12	1525	1451	566	886	61	105	0.63	8,3	7,0	9.90	147
2/12	Diet: Bread and milk										
2/13	Bled 363 cc.										
2/14	Bled 363 cc.										
2/15	385	837	560	276	33	46	0.70	3,3	7,4	9.80	85
2/15	Diet: Beef heart										
2/21	500	980	647	333	34	51	0.77	3,3	10,8	9.70	101
2/28	602	885	523	363	41	68	0.68	5,0	19,8	9.80	90
3/7	840	1077	560	517	48	78	0.56	6,9	19,8	9.80	109
3/14	904	1062	531	531	50	85	0.57	7,5	14,0	9.30	114
3/21	1235	1272	585	687	54	93	0.60	7,8	10,0	9.50	134
3/28	1146	1180	543	638	54	97	0.58	8,3	8,0	9.50	124
4/5	1280	1243	572	671	54	103	0.56	9,2	8,6	9.30	134
4/11	1478	1342	604	738	55	110	0.58	9,4	18,6	8.80	152

Experimental history, see table 46-b.

liver show up to advantage as compared with the mixed diet under such conditions. Apparently the fasting or sugar feeding or other limited diet causes a draining of the body's reserve and subsequent blood regeneration suffers because of this depletion of reserve or impairment of function.

Tables 56 and 57 are incomplete in that the amount of beef heart is not known but the weights give assurance that a liberal amount of food

was consumed. There is only a trifling loss of weight during the last week of the experiment when the blood picture had returned to normal. It is obvious that the blood regeneration in these two experiments is much slower than that recorded in experiments 53 and 54. Four to 6 weeks elapse before the hemoglobin and hematocrit return to normal and 5 to 7 before the pigment volume and red cell count return to the original level.

TABLE 57

Blood regeneration—beef heart. Dog 17-192. Bull mongrel, male, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
4/24	1157	1032	537	496	48	112	0.78	7,2	10,6	9.80	105
4/24	Diet: Bread and milk										
4/25	Bled 258 cc.										
4/26	Bled 258 cc.										
4/27	444	822	633	189	23	54	0.84	3,2	12,8	9.50	87
4/27	Diet: Beef heart										
5/2	662	883	609	274	31	75	0.79	4,7	11,6	9.90	89
5/9	804	992	585	407	41	81	0.67	6,0	8,0	9.50	104
5/16	1030	1050	567	483	46	98	0.80	6,1	12,0	9.50	110
5/23	1255	1172	633	539	46	107	0.73	7,3	9,0	9.50	123
5/30	1308	1147	585	562	49	114	0.81	7,0	8,0	9.50	121
6/6	1360	1214	607	607	50	112	0.64	8,7	6,8	9.10	133

No previous anemia experiments on this dog.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

Tables 58 and 59 both deal with bile fistula dogs. The experiments were performed at the same time under identical conditions and the reaction to the beef heart diet is strikingly uniform. It is known from the autopsy notes that the bile was completely excluded from the intestine. A meat diet is not well tolerated by these bile fistula dogs over long periods and a loss of weight is usually noted under such conditions. As a result of this long period of meat feeding we note subsequent intoxication which resulted fatally in spite of a mixed diet régime.

The beef heart was eaten with relish but the amounts given are not recorded. There is a distinct gain in hemoglobin, red cells and pigment volume during each of the first 3 weeks. The level at the end of

TABLE 53

Blood regeneration—beef heart—bile fistula. Dog 17-35. Bull mongrel, female, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
5/28	1330	1244	560	684	55	107	0.74	7,2	20,0	9.10	136	
5/29	Bled 311 cc.											
5/30	Bled 311 cc.											
5/31						48	0.77	3,1	45,6	9.10		
5/31	Diet: Beef heart											
6/8	540	900	567	333	37	60	0.79	3,8	14,6	8.20	109	No increase in bile pigment
6/15	868	923	480	443	48	94	0.78	6,0	7,0	8.40	109	
6/22	952	933	476	457	49	102	0.72	7,1	8,4	8.10	115	
6/29	800	1000	590	410	41	80	0.74	5,4	6,8	8.40	119	
7/6	649	729	459	277	38	89	0.78	5,7	7,0	8.00	91	
7/9	Diet: Mixed diet											
7/13	694	846	533	313	37	82	0.77	5,3	20,0	8.80	96	
7/18	797	848	500	348	41	94	0.90	5,2	19,8	9.00	94	
8/9	Death from bile fistula intoxication											

Bile pigment daily output per 6 hours before this experiment (28 day average) = 14.0 mgm.

Bile pigment daily output per 6 hours during this experiment (30 day average) = 13.4 mgm.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

the third week is almost normal in both dogs and if the experiments had been terminated at this point we should of necessity conclude that the reaction was similar to that so often observed in the normal dog.

But the fourth week in both dogs shows a decided drop in pigment volume, hemoglobin and red cell hematocrit.

We fortunately have the daily *bile pigment output figures* at hand and can say that there is no increase in bile pigment elimination during this week of falling hemoglobin. So we may not explain this decrease in the curve as due to some agent destructive to the red cells. It has been established (2) that a sudden destruction of red cells in the blood stream will result in an increased output of bile pigment although the reaction is not in any degree a quantitative reaction as some observers have claimed (3). We have no convincing explanation for these observed facts but suggest that the poor quality of the red cell may be a

TABLE 58-B

Experimental history. Dog 17-35 (bile fistula)

EXPERIMENT	DIET	BLOOD REGENERATION		WEIGHT	REMARKS
		Pigment volume	Blood per kilogram		
Begin 12/18/16 Bled 600 cc. End 1/17/17	Lean meat			<i>kgm.</i>	
				8.80	100 Hb.
				8.90	(4 bleedings)
		892	99	8.90	101 Hb.
3/8/17	Bile fistula operation				
Begin 5/28/17 Bled 622 cc. End 7/6/17	Beef heart	1330	136	9.10	107 Hb.
				9.10	
		649	91	8.00	Maximum regeneration 3 weeks

factor. Limited diets as well as splenectomy under certain experimental conditions seem to be associated with red cells which are prone to disintegrate more readily than normal. .

The average daily bile pigment output is given in each table for a 30-day period before the experiment and during the anemia regeneration period. One dog shows identical figures for the mixed diet control and beef heart period. The other dog shows a much higher output on the mixed diet than on the beef heart diet. We believe this is to be explained by the mixed diet which is made up of meat scraps, bones, bread, potatoes, table scraps, etc., and is given in moderate excess. This allows a certain choice on the part of the dog and if the animal prefers the carbohydrate fractions we may observe the familiar reac-

tion to carbohydrate feeding in the bile fistula dog, namely, an increase in bile pigments.

TABLE 59

Blood regeneration—beef heart—bile fistula. Dog 17-155. Bull mongrel, male, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
5/28	1560	1416	708	708	50	110	0.67	8,2	11,2	11.50	123	
5/28	Diet: Mixed diet											
5/29	Bled 354 cc.											
5/30	Bled 354 cc.											
5/31	495	935	645	290	31	53	0.63	4,2	9,2	10.80	86	
5/31	Diet: Boiled beef heart											
6/8	1030	1213	752	461	38	85	0.70	6,1	9,0	10.90	111	No bile pigment increase.
6/15	1165	1265	645	620	49	92	0.66	7,0	10,4	10.90	116	
6/22	1385	1281	615	666	52	108	0.68	7,9	9,8	10.00	128	
6/29	946	1186	700	484	41	80	0.67	6,0	14,6	9.80	121	
7/6	1100	1038	571	468	45	106	0.73	7,3	15,0	10.00	103	No bile pigment increase
7/13	1138	1138	683	455	40	100	0.86	5,8	14,4	9.90	115	
7/18	1120	1288	824	464	36	87	0.84	5,2	9,8	10.30	125	
7/19	Diet: Mixed diet											
8/9	Death from bile fistula intoxication											

Bile pigment daily output per 6 hours before this experiment (30 day average) = 21.8 mgm.

Bile pigment daily output per 6 hours during this experiment (30 day average) = 15.2 mgm.

No previous anemia experiments on this dog.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

We may recall that the fasting bile fistula dog will react as promptly to anemia as the normal dog (table 23) and the bile fistula dog on a bread and milk diet also presents a normal blood regeneration curve

(table 32). But the meat diet reaction in the bile fistula dog is not like the reaction of the normal animal. The unfavorable clinical reaction of the bile fistula dog to the meat diet is often conspicuous

TABLE 60

Blood regeneration—liver. Dog 19-83. Bull mongrel pup male

DATE, 1918-1919	PIGMENT VOLUME Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
12/2/18	1090	1001	540	466	46.1	108	0.59	9,1	8,8	9.30	109	R. B. C. small
12/2	Diet: Bread and milk											
12/3	Bled 250 cc.											
12/4	Bled 250 cc.											
12/6	489	790	586	202	25.5	62	0.63	4,9	13,8	8.80	90	* Slight
12/7	Bled 200 cc.											
12/9	630	1032	770	258	25.0	61	1.20		13,7	8.65	119	
12/9	Diet: 550 grams cooked beef liver†—100 calories per kilo											
12/16	873	910	536	370	40.6	96	0.94	5,1	11,4	8.85	103	
12/23	962	962	550	407	42.3	100	0.56	8,9	19,2	9.35	103	
12/30	1304	1100	566	530	48.1	118				9.90	111	
12/30	Diet: Mixed diet											
1/8/19	1300	1235	642	580	47.0	105	0.80	6,6	14,2	10.75	115	
1/15	1095	1200	670	519	43.2	91	0.75	6,1	12,8	11.40	105	
1/22	1355	1300	685	600	46.2	104	0.75	6,9	8,0	11.30	115	

*Poikilocytosis of red blood cells.

† Beef liver cooked, fat and connective tissue removed, ground.

No previous anemia experiments on this dog.

(diarrhea and loss of weight and activity) and this may explain the observations recorded in tables 58 and 59.

Tables 60 and 61 show the remarkable influence which cooked liver exerts upon blood regeneration. As the sole article of food cooked liver may not be well tolerated, but in these two experiments the dogs

ate all of the liver and gained weight. The remarkable gain in hemoglobin, red cells and pigment volume is at once obvious at a glance. One experiment (table 61) shows practically complete regeneration in 2 weeks from the usual anemia level and both dogs are more than

TABLE 61

Blood regeneration—liver. Dog 18-114. Bull mongrel, female, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
11/14	1640	1411	643	763	54.0	116	0.58	11,3	14,8	13.50	105
11/14	Diet: Crackermeal and milk										
11/15	Bled 353 cc.										
11/16	Bled 353 cc. No distress										
11/18	852	1077	695	372	34.5	79				12.85	84
11/18	Bled 270 cc. No distress										
11/20	592	1015	724	280	27.6	58	0.82	3,5	14,2	12.50	81
11/20	Diet: 750 grams cooked beef liver*										
11/27	1305	1290	726	540	41.8	101	0.74	6,8	11,8	13.25	97
12/4	1730	1480	740	720	48.5	117	0.76	7,7	20,0	13.35	111
12/11	1902	1516	728	766	50.5	125	0.82	7,6	13,4	13.65	111
12/13	Diet: Mixed diet										
12/20	1479	1333	713	613	46.0	111	0.65	8,6	14,6	13.90	96
12/27	1295	1276	670	594	46.5	101				13.75	93
1/10/19	1625	1390	668	716	51.5	117	0.68	8,6	12,8	14.20	98

* Beef liver cooked, fat and connective tissue removed, ground.
Experimental history, see table 12-b.

back to normal in 3 weeks. We are able to refer to a number of other experiments on this dog (table 61, dog 18-114, exper. history table 12-b) to give a good line on the type normal blood regeneration.

Table 62 shows another experiment with cooked beef liver but the amount of liver fed is much less and the rest of the food caloric value

is made up by crackermeal and milk. We see this small amount of liver causing a prompt rise of the hemoglobin and red cells to normal in 3 weeks, whereas the crackermeal and milk alone would require at least 5 to 6 weeks for complete regeneration.

TABLE 62

Blood regeneration—liver and crackermeal. Dog 19-15. Brindle bull mongrel, female, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
8/9	1702	1120	462	650	58.0	152	0.95	7,9	6,2	10.85	103	
8/9	Diet: Crackermeal and milk											
8/12	Bled 280 cc. No distress											
8/13	Bled 280 cc. No distress											
8/15	663	762	501	253	33.2	87				10.40	73	
8/15	Bled 190 cc. No distress											
8/17	468	669	493	173	25.8	70	0.92	3,8	16,8	10.35	65	* Poik. ++
8/17	Diet: 70 grams cooked beef liver, † 200 grams crackermeal, 500 cc. milk											
8/23	664	874	517	267	30.5	76	0.88	4,3	15,0	10.85	81	
8/30	1290	949	466	478	50.3	136	0.90	7,5	15,8	11.30	84	
9/6	1600	1096	504	588	53.6	146	0.92	7,9	8,2	11.20	98	
9/13	1700	1089	495	594	54.5	156	0.96	8,1	10,8	11.50	95	

* Poikilocytosis of red blood cells.

† Beef liver cooked, fat and connective tissue removed, ground.

No previous anemia experiments on this dog. See table 64 for subsequent experiment.

Table 63 is not very satisfactory but is included because several interesting points may be made. This dog at the beginning of the experiment was very young, approximately 4 months, but the date of birth was not positively known. The pup had a hemoglobin of 73 per cent, which is not unusual in young dogs of this age. The amounts bled were less than normal because of this fact, but the total reserve

was evidently considerable, as indicated by the high figures after the bleeding. The young dog was growing rapidly during the whole period of the experiment a gain of 3 kilos body weight in 6 weeks. The blood volume shows a considerable increase during this period, almost 100 per cent gain. The gain in hemoglobin and hematocrit is steady and

TABLE 63

Blood regeneration—liver and bread. Dog 18-114. Bull mongrel pup, female

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
4/24	553	758	455	291	38.4	73	0.53	6,9	23,6	7.85	94
4/24	Diet: Bread and milk										
4/26	Bled 189 cc.										
4/27	Bled 90 cc. No distress										
4/29	435	805	528	255	31.7	54	0.58	4,7	14,6	7.65	105
4/30	Diet: 200 grams bread, 500 cc. milk, 50 grams cooked beef liver,* ground up with bread										
5/8	599	740	432	293	39.6	81	0.56	7,2	8,4	8.00	93
5/15					43.6	88	0.52	8,5	20,6	8.80	
5/22	825	938	514	411	43.8	87	0.54	8,0	16,0	9.50	98
5/29	791	807	463	330	40.9	98	0.69	7,1	22,8	9.60	84
5/29	Diet: Mixed diet										
6/5		902	497	391	43.4					10.45	86
6/10	1059	1009	556	443	43.9	105	0.67	7,8	11,8	10.70	94

* Beef liver cooked, fat and connective tissue removed, and ground up with bread.

Experimental history, see table 12-b.

No previous anemia experiments on this dog.

comes close to the average normal at the end of the experiment, in marked contrast to the level at the beginning of the experiment.

Meat extract (tables 64 and 65) evidently does not add anything to a given diet which in itself is especially favorable to blood regeneration. We have no evidence to show that meat extract is favorable or unfa-

avorable to blood reconstruction. Table 64 shows the rapid blood regeneration which we expect on a cooked liver diet and the return to normal requires but 3 weeks. We note again in this experiment the fall which often occurs following a sudden change to another diet

TABLE 64

Blood regeneration—liver and beef extract. Dog 19-15. Brindle bull mongrel, female, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
11/14	1615	1260	506	746	59.3	128	0.76	8,4	10,8	12.40	102
11/14	Diet: Crackermeal and milk										
11/15	Bled 315 cc. No distress										
11/16	Bled 315 cc. No distress										
11/18	584	808	550	255	31.5	72				11.95	68
11/18	Bled 202 cc.										
11/20	508	796	568	224	28.1	64	0.69	4,6	14,4	11.35	70
11/20	Diet: 450 grams cooked beef liver* and 10 grams Liebig's beef extract										
11/27	808	917	565	347	37.9	88	0.76	5,8	12,8	11.30	81
12/4	1100	1002	555	458	45.0	108	0.66	8,2	15,3	11.50	89
12/11	1300	1034	560	518	47.8	120	0.60	10,3	12,2	11.35	95
12/13	Diet: Mixed diet										
12/20	912	954	550	395	41.6	96	0.56	8,6	16,8	11.95	80
12/27	926	1010	564	427	42.2	92				11.85	85
1/10/19	1122	1060	540	508	48.0	106	0.91	8,0	13,6	12.00	88

* Beef liver cooked, fat and connective tissue removed, ground.

Refer to table 62 for previous experiment.

(mixed diet) which, too, is very favorable for blood regeneration and maintenance. It is clear that there is no fluctuation in plasma volume which would supply an easy explanation for this phenomenon.

Meat extract (table 65) does not modify the reaction which may be expected following a liberal bread and milk diet. The blood picture is

returned almost to normal in 4 weeks after the anemia and there is a gain in weight of 1 kilo. A change to a mixed diet gives a favorable reaction as is usual under these circumstances. We may refer also to a part of table 66, which gives more data on the influence of bread and

TABLE 65

Blood regeneration—meat extract—bread and milk. Dog 18-116. Bull mongrel pup, female

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
4/24	903	962	459	486	50.5	94	0.65	7,2	9,8	9.75	99	* Slight
4/24	Diet: Bread and milk											
4/25	Bled 240 cc. from jugular vein											
4/26	Bled 190 cc. from jugular vein											
4/29	422	862	594	259	30.0	49	0.57	4,3	10.2	9.65	89	* Slight
4/29	Bled 110 cc. from jugular vein											
4/30	Diet: 200 grams bread, 500 cc. milk, 10 grams Liebig's beef extract											
5/8	717	956	591	347	36.3	75	0.48	7,8	10,0	9.60	99	*
5/15	882	1297	760	517	39.9	68	0.46	7,3	8,6	9.50	137	*
5/22	859	1035	594	430	41.5	83	0.51	8,2	22,8	10.70	97	*
5/29	865	961	550	403	41.8	90	0.54	8,3	10,2	10.85	89	*
5/29	Diet: Mixed diet											
6/10	1207	1128	634	483	42.8	107	0.63	8,5	30,0	12.50	90	
6/18						104						

* Poikilocytosis of red blood cells.

Experimental history, see table 13-b.

milk plus meat extract. The meat extract adds nothing to the reaction which is identical with the expected reaction from the bread and milk alone.

Tables 66 and 67 are to be considered together. The experiments were done at the same time under identical conditions and the results

TABLE 66

Blood regeneration—watery liver extract and sugar; bread and milk; meat extract, bread and milk; beef heart, bread and milk. Dog 17-157. Coach mongrel, female, young adult

young adult

DATE, 1919	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
2/20	1340	1180	567	606	51.4	113	0.79	7,2	8,2	10.35	114	
2/20	Diet: Bread and milk											
2/21	Bled 295 cc. No distress											
2/22	Bled 295 cc. Distress. Injected 50 cc. of 5 per cent sugar solution											
2/24	420	806	602	198	24.6	52	0.96	2,7	8,6	9.50	85	
2/26	Diet: 100 grams sugar, 10 grams watery liver extract,† 250 cc. water											
3/5	720	868	519	345	39.7	83	0.90	4,6	11,0	8.95	97	
3/12	700	813	493	312	38.4	86	0.88	4,9	7,2	8.25	99	
3/18	666	730	420	294	40.2	91	0.80	5,7	8,8	8.05	91	
3/18	Diet: 200 grams bread, 300 cc. milk											
3/26	824	929	560	360	38.7	89	0.85	5,2	10,0	8.40	110	
3/26	Diet: 200 grams bread, 300 cc. milk, 10 grams commercial meat extract											
4/2	756	913	556	398	38.1	83	0.90	4,6	10,8	8.75	104	*
4/8	635	829	522	298	36.0	77	0.67	5,7	14,0	8.75	95	
4/14	714	876	538	324	37.0	82	0.73	5,6	11,2	8.75	100	
4/21	860	984	584	389	39.6	87	0.72	6,0	12,8	8.90	110	
4/21	Diet: 200 grams beef heart (cooked), 200 grams bread, 300 cc. milk											
4/28	772	942	588	345	36.6	82	0.67	6,1	12,4	9.55	99	
5/7	842	961	572	384	40.0	88	0.73	6,0	15,4	10.00	96	
5/12	890	998	590	397	39.8	89	0.72	6,2	15,0	9.90	101	
5/12	Diet: Mixed diet											

* Poikilocytosis of red blood cells.

† Watery liver extract: Beef liver cut up into small cubes, allowed to stand in water over night in ice-chest, boiled in same water, and filtered. Filtrate concentrated to thick paste.

TABLE 66-B
Experimental history. Dog 17-157

EXPERIMENT NUMBER	DIET	BLOOD REGENERATION		WEIGHT * <i>kgm.</i>	REMARKS
		Pigment volume	Blood per kilogram		
Begin 9/3/17	Crackermeal,	1025	115	8.40	
Bled 496 cc.	lard and gela-	380	83	8.20	
End 10/25/17	tin	890	102	8.70	
Begin 3/6/18	Crackermeal,	1084	99	9.80	Table 71
Bled 488 cc.	lard, butter	614	90	9.10	
End 4/9/18	and Blaud's pills	984	106	8.30	
Begin 5/20/18	Sugar, metabol-	1113	120	10.20	
Bled 612 cc.	ism, desiccated	313	75	9.50	
End 6/18/18	beef heart	663	100	7.60	
					Maximum regen- eration 3 weeks. Pigment vol- ume 698 cc.
Begin 8/28/18	Hb. and sugar	1366	102	10.10	Table 78 (3 bleedings) Pigment volume 805 at end of Hb. period
Bled 710 cc.	feeding	433	79	9.25	
End 11/12/18	Hb. intravenous- ly and sugar feeding	886	114	8.15	
	Hb. intravenous- ly and cracker- meal + milk				
	Crackermeal, milk and dried yeast				
Begin 2/20/19	Liver extract,	1340	114	10.35	Table 66
Bled 590 cc.	sugar	420	85	9.50	
End 5/12/19	Bread, milk, meat extract	890	101	9.90	
	Beef heart, bread and milk				Pigment volume 666 cc. at end of sugar feeding

are very suggestive. Both dogs have been observed in many other experiments and their anemia reactions are therefore well known. One dog was given the watery extract of beef liver and the other the liver residue. Sugar, 100 grams, was added to each feeding and it is clear

TABLE 67

Blood regeneration—liver residue and sugar; bread and milk; crackermeal and bread and milk; cooked beef liver and bread and milk. Dog 16-158. Coach mongrel, male, young adult

DATE, 1919	PIGMENT VOLUME Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	P. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
2/20	1862	1375	549	820	59.6	135	0.77	8,8	7,0	12.35	111	
2/20	Diet: Bread and milk											
2/21	Bled 344 cc. No distress											
2/22	Bled 344 cc. Dyspnea; injected 50 cc. of 5 per cent sugar solution											
2/24	823	983	625	353	35.9	84	1.02	4,1	19,6	11.40	86	
2/25	Bled 246 cc.											
2/27	478	778	562	211	27.2	61	0.85	3,6	8,4	11.10	70	
2/27	Diet: 100 grams sugar, 200 cc. water by stomach tube; 100 grams liver residue†											
3/5	692	867	531	331	38.2	80	0.76	5,3	6,4	10.75	81	*
3/12	876	900	517	373	41.5	97	0.84	5,8	5,4	9.95	90	* Poik.+
3/18	738	754	421	329	43.7	98	0.83	5,9	14,4	9.15	82	* Poik.+
3/18	Diet: 200 grams bread, 300 cc. milk											
3/26	725	923	597	316	34.3	79	0.77	5,1	6,8	10.15	91	* Poik.
3/26	Diet: 200 grams bread, 100 grams crackermeal, 300 cc. milk											
4/2	748	944	600	339	35.9	79	0.61	6,5	9,8	10.50	90	* Poik.+
4/8	685	966	616	340	35.2	71	0.61	5,8	10,8	11.10	87	* Poik.
4/14	762	968	608	355	36.7	79	0.59	6,7	7,8	12.75	76	* Poik.
4/21	840	1072	640	406	37.9	78	0.56	7,0	10,6	11.20	96	
4/21	Diet: 200 grams cooked beef liver, 200 grams bread, 300 cc. milk											
4/28	820	1027	629	388	37.8	80	0.73	5,5	12,0	11.95	86	
5/7	1155	1155	595	549	47.5	100	0.75	6,7	9,6	11.45	101	
5/12	1074	1095	583	502	45.8	98	0.66	7,4	13,0	11.80	93	* Poik.+
5/12	Diet: Mixed diet											

* Poikilocytosis of red blood cells.

† Liver residue: Residue left after water and alcoholic extraction; put in meat press and all liquid removed. Just before feeding residue was again washed and brought to boiling point to remove all traces of alcohol. Dog did not always eat full amount of food mixture.

Experimental history, see table 46-b.

that both the liver watery extract and liver residue exert a certain influence upon the blood regeneration which is much more than can be accounted for by the sugar alone. The liver residue has greater influence upon the blood regeneration than does the liver watery extract but the difference is not striking.

Bread and milk feeding for 1 week subsequent to these sugar and liver periods does not cause much reaction. There is a slight loss in hemoglobin and red cell hematocrit in experiment 67 (liver residue).

The next 4 weeks are similar and bread or crackermeal and milk are the essential features of the diet. The level of pigment volume, red cell hematocrit and hemoglobin is constant. There is slight gain in the red cell counts.

We have pointed out the fact that a prolonged diet period unfavorable to hemoglobin regeneration will leave the dog in a condition which may be clinically excellent but from the standpoint of formation of hemoglobin very unfavorable. These two dogs were still anemic although in excellent physical condition and of practically normal weight. Under such circumstances we feel that any diet is given its most severe test and few diets of limited nature can give a favorable reaction as regards blood regeneration. Under ordinary circumstances a diet of beef heart, bread and milk (table 66) is favorable for blood regeneration but under this severe test with unfavorable conditions we note little if any blood regeneration during a period of 3 weeks.

Cooked beef liver with bread and milk even under these same unfavorable conditions is able to effect a prompt blood regeneration—conspicuous in red cell hematocrit, red count and hemoglobin. This is the severest test of any diet factor in its relation to blood regeneration. Also refer to table 22 (same experiment).

DISCUSSION

The reader will observe many individual differences in the reactions of various dogs to a unit type of secondary anemia. Some of these vagaries we are as yet unable to explain but others are now much clearer than in the earlier stages of this investigation. We have noted that on occasions certain dogs *made anemic* for the *first time* presented a most unusually rapid regeneration, even on a very limited diet. This is not the rule, but is sufficiently common to suggest caution in conclusions drawn from such experiments. A repeat experiment will

give the true constant reaction. How to explain this fact is not clear to us, but a simple way out is to assume a reserve present in the body under these conditions which permit of unusual blood regeneration even under most unfavorable diet conditions. A knowledge of this fact may keep the investigator from falling into error in deductions drawn from single experiments. With this exception the dogs will show uniform reactions when we repeat anemia experiments under uniform conditions.

It is clear that long limited diet periods following the standard anemia may preserve the dogs in excellent physical condition as concerns weight, general condition and activity. These same dogs, however, may continue to present a definite anemia. Under such conditions many diet mixtures are unable to stimulate any blood regeneration. Any diet factors are put to the severest test when they are administered to dogs under such circumstances and we are inclined to accept this as the severest test for any given diet factor. Cooked liver gives a very favorable reaction and causes blood regeneration even under these severe test conditions.

Following a simple anemia many diet mixtures, if given at once, will cause a distinct gain in hemoglobin and red cells. But if a limited diet period intervenes between the anemia and the exhibition of the test diet we will see a negative reaction. This may be illustrated by bread and milk given in *liberal amounts* sufficient to permit a gain in body weight or at least a maintenance of body weight. If the dog is bled and at once placed on a bread and milk diet there will usually appear a slow steady gain in pigment volume. If the same dog is placed on a limited *carbohydrate diet* for 3 to 4 weeks before being changed to a liberal bread and milk diet we will usually observe subsequently an unchanged level of pigment volume, red cell hematocrit and hemoglobin. Under such unfavorable conditions the bread and milk diet is unable to give a favorable reaction for the blood regeneration. In certain experiments the cooked beef heart is also unable to modify the curve of blood regeneration under these unfavorable conditions. Cooked liver is able to induce blood regeneration even under the most unfavorable conditions. The same is true for the common *mixed diet* of table scraps. It is important to keep these facts in mind when we evaluate the reaction following the administration of a given diet factor under different experimental conditions.

The question at once confronts us: what part of the meat or liver substance is responsible for the favorable blood reaction? First we

must investigate the pigment substances present in the meat—for example, the hemoglobin and myohematin. Some experiments with hemoglobin appear in the next paper of this series, and we hope soon to report other experiments dealing with the myohematin pigment.

SUMMARY

Cooked lean beef and beef heart are diet factors of importance as regards blood regeneration subsequent to simple secondary anemia. These food substances alone or in combination with other foods will give a rapid blood regeneration after anemia.

Anemia is produced by bleeding one-fourth of the measured blood volume on each of 2 successive days. This anemia will be completely repaired within 3 to 4 weeks if the dog is given a liberal diet of meat or beef heart.

Cooked liver is as sufficient as meat and may be even more efficient in promoting complete blood regeneration subsequent to a standard anemia. Blood regeneration may be completed in 2 to 4 weeks.

Commercial meat extract is inert and watery liver extract has but little influence upon blood regeneration.

The meat diet reaction in the bile fistula dog is not exactly like the reaction of the normal animal.

BIBLIOGRAPHY

- (1) MENDEL AND OSBORNE: *Proc. Soc. Exper. Biol. and Med.*, 1918, xv, 71.
- (2) WHIPPLE AND HOOPER: *This Journal*, 1917, xliii, 258.
- (3) BRUGSCH AND YOSHIMOTO: *Zeitschr. f. exper. Path. u. Therap.*, 1910, viii, 639.

BLOOD REGENERATION FOLLOWING SIMPLE ANEMIA

V. THE INFLUENCE OF BLAUD'S PILLS AND HEMOGLOBIN

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The familiar fact that iron in some form is very frequently given in cases of secondary anemia made it imperative to test this drug under a variety of experimental conditions. The outstanding fact in our experiments is that *iron given as Bland's pills has no influence in secondary anemia under the conditions of these experiments*. It may be objected that the Bland's pills were not fresh or were not dissolved in the dog's intestinal tract. We obtained these Bland's pills from a large wholesale firm in this city and they were soft and easily crushed into a soft, pasty material. The pills were crushed before being given by mouth. Further objections may be made that this drug has no influence on the dog but does have potency when administered to human beings. This of course is not subject to proof, but the claims for the potency of iron in conditions of secondary anemia do not stand on firm ground. We invite attention to the profound influence which is properly attributed to diet factors. Those who claim that iron is a potent drug must exclude the food factors which are known to be concerned before they can prefer too many objections to our negative results.

The feeding of blood has at times been used in the treatment of secondary anemia. We are able to find some experimental evidence to support this treatment, but whole red cells or hemoglobin given by mouth in the form of a dry powder do not appear to influence profoundly the blood regeneration curve. Our experiments show that hemoglobin does have a distinct influence on blood regeneration but not sufficient to warrant its use in uncomplicated secondary anemia in view of the favorable reactions due to meat and other diet factors.

The favorable reaction which seems to accompany administration of hemoglobin by injection (intravenous and intraperitoneal) may be

of some value in the treatment of certain forms of anemia. It is possible that the reaction to this type of injection may differ from that associated with a transfusion and in certain diseases this procedure (hemoblobin injection) may stimulate rather than depress the bone marrow. Further experimental work is in progress.

EXPERIMENTAL OBSERVATIONS

The same technical procedures are used in these experiments as have been described in the first paper of this series. Bland's pills were given daily. The experimental histories give the complete list of anemia experiments on any given dog and are referred to in each experiment. The control experiments are frequently given in the other papers of this series, but the proper reference is appended to the experiment dealing with iron or hemoglobin. We hope to report experiments in the near future dealing with other drugs used in the treatment of anemia. We expect to test many other pigment substances besides hemoglobin as to their influence on blood regeneration. This includes animal and vegetable pigments as they occur in various meats, fish and vegetables.

Table 68 represents a long anemia experiment with Bland's pills which is conclusive in showing the complete failure of this drug (carbonate of iron) to influence blood regeneration under the conditions of the experiment. Subsequent experiments will make it clear that *Bland's pills are inert* in so far as any influence on this type of anemia is concerned.

The experimental history of this dog (table 18-b) gives the reaction of this animal to other diet factors and establishes the type reaction to secondary anemia. It is fair to say that this same type of blood regeneration would be expected without the influence of Bland's pills. This dog is more resistant than usual and tolerated this limited diet for a long period without any symptoms of dietary deficiency disease. The falling hemoglobin, red cell count and weight curves, however, may indicate an impending dietary deficiency complex which did not develop because of the change to a liberal mixed diet.

The Bland's pills were given daily and crushed before administration by mouth. Sufficient bread and skim milk were given to maintain the body weight constant until the last 2 weeks. During the first 8 weeks the red cell hematocrit is stationary but the hemoglobin rises slowly. During this period the red count rises slowly and uniformly

TABLE 68

Blood regeneration—bread, milk and Blaud's pills. Dog 16-169. Bull mongrel, female, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
2/12	939	978	489	489	50	96	0.73	6,6	6,2	7.70	127	
2/12	Diet: Bread and milk											
2/13	Bled 244 cc.											
2/14	Bled 244 cc.											
2/15	267	580	423	157	27	46	0.77	3,0	6,8	7.40	80	
2/15	Bread, milk and 2 Blaud's pills daily											
2/21	348	809	566	243	30	43	0.86	2,5	10,8	7.50	108	
2/28	375	694	493	201	29	54	0.71	3,8	7,4	7.40	94	
3/7	393	667	487	180	27	59	0.69	4,3	5,6	7.30	91	*
3/14	428	738	524	214	29	58	0.66	4,4	11,0	7.20	104	*
3/21	335	697	495	202	29	48	0.52	4,6	7,4	6.80	103	*
3/28	312	625	431	194	31	50	0.48	5,2	10,0	7.00	89	*
4/5	382	694	486	208	30	55	0.48	5,7	6,8	6.80	102	*
4/11	368	681	470	211	31	54	0.46	5,9	13,6	7.00	97	*
4/18	500	725	471	254	35	69	0.51	6,7	11,2	7.30	99	*
4/25	576	728	480	258	35	78	0.51	7,7	5,6	7.20	102	*
5/4	560	700	455	245	35	80	0.52	7,7	8,6	7.00	100	*
5/11	617	771	455	316	41	80	0.53	7,6	12,4	7.40	104	*
5/18	624	762	457	305	40	82	0.55	7,4	9,4	7.20	106	*
5/25	688	819	467	352	43	84	0.57	7,3	6,6	7.00	117	*
6/1	716	721	418	303	42	99	0.59	8,4	6,6	6.80	106	*
6/8	830	847	491	355	42	98	0.64	7,6	5,4	7.00	121	*
6/15	833	833	500	333	40	100	0.66	7,6	5,8	6.50	127	*
6/27	705	839	520	319	38	84	0.62	6,8	10,0	6.50	129	*
7/11	477	597	394	203	34	80	0.67	6,0	7,6	6.20	96	*
7/16	456	570	393	177	31	80	0.74	5,4	6,2	5.90	97	*

* Marked fragmentation of red blood cells.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

Experimental history, see table 18-b.

and continues this rise to a figure even above normal. It is noted, however, that there was marked fragmentation of the red cells and we can scarcely account for this great increase in red cells (2,500,000 to 8,400,000) with the red cell hematocrit showing only a rise from 27 per cent to 42 per cent, except on the ground of red cell fragmentation or abortive red cell construction. The hemoglobin does not keep pace

TABLE 69

Blood regeneration—bread, milk and Blaud's pills. Dog 17-193. Bull mongrel, female, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
4/24	1089	1100	517	584	53	99	0.75	6,6	15,8	8.40	131	
4/24	Diet: Bread and milk											
4/25	Bled 275 cc.											
4/26	Bled 275 cc.											
4/27	333	710	547	163	23	47	0.87	2,7	24,0	8.20	87	
4/27	Diet: Bread, milk and 2 Blaud's pills daily											
5/2	458	683	492	191	28	67	0.82	4,1	9,4	8.00	85	
5/9	636	795	493	302	38	80	0.75	5,3	9,4	8.00	99	
5/16	856	1006	533	473	47	85	0.76	5,6	6,6	8.00	102	
5/23	1008	1039	561	478	46	97	0.69	7,0	7,4	8.00	129	*
5/30	1041	1021	521	500	49	102	0.67	7,6	12,4	8.20	124	*
6/6	1055	1014	527	487	48	104	0.65	8,0	6,2	7.90	128	*

* Fragmentation of red blood cells.

No previous anemia experiments on this dog.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

with the red count and a fall in the color index from 77 per cent to 46 per cent is recorded.

The high water mark for blood regeneration is noted after 3½ months and the level at this time is far from normal. Subsequently there is a loss in red cell hematocrit, red count, hemoglobin and pigment volume. We believe this indicated a tendency toward a dietary deficiency dis-

case which would have developed had the bread and milk diet been continued. The plasma volume as usual is constant throughout the entire experiment with the exception of the last 2 weeks.

The next experiment (table 69) shows a fairly complete blood regeneration during a period of 5 weeks on a bread and milk diet plus Blaud's pills. It is to be observed that no previous anemia experi-

TABLE 70

*Blood regeneration—crackermeal, lard, butter, milk powder and Blaud's pills.
Dog 17-205. Bull mongrel, male, young adult*

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
3/4	1182	1055	538	518	49	112	0.70	8,0	10,8	12.60	84	
3/4	Diet: 279 grams crackermeal, 10 grams lard, 10 grams butter											
3/6	Bled 274 cc.											
3/7	Bled 254 cc.											
3/9	605	840	605	235	28	72	0.82	4,4	30,4	12.1	69	*Anis.
3/9	Diet: 142 grams crackermeal, 10 grams lard, 10 grams butter, 279 grams milk powder, 2 Blaud's pills daily											
3/15	565	807	573	234	29	70	0.88	4,0	27,4	11.40	71	*Anis.
3/20	798	928	622	306	33	86	0.93	4,6	9,2	11.20	83	*Anis.
3/27	682	802	545	256	32	85	0.79	5,4	19,2	10.90	73	*Anis.++
4/3	983	919	597	323	35	107	0.82	6,5	21,6	10.90	84	*Anis.++
4/9	795	750	480	270	36	106	0.79	6,7	7,4	9.60	78	*Anis.++
4/9	Dietary deficiency disease. Recovery.											

* Anisocytosis of red blood cells.

Refer to table 34 for control.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

ments had been done on this dog and the capacity for blood regeneration may be greater under such circumstances. The reaction is not unusual, however, in view of the liberal amounts of bread and milk which were sufficient to maintain the body weight. It cannot be granted that the Blaud's pills had any influence upon the blood regeneration.

Table 70 is of considerable interest because we are able to refer to a control period (table 34) on a similar diet but without the Bland's pills. If anything the control period shows slightly more blood regeneration during the first 5 weeks. The control experiment was of much longer duration (12 weeks) without dietary deficiency disease symptoms. This may be due to the fact that this control experiment (table 34)

TABLE 71

*Blood regeneration—crackermeal, lard, butter and Bland's pills. Dog 17-157.
Coach mongrel, female, young adult*

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
3/4	1084	976	537	439	45	111	0.82	6,8	8,4	9.80	99	
3/4	Diet: 199 grams crackermeal, 10 grams lard, 10 grams butter											
3/6	Bled 254 cc.											
3/7	Bled 234 cc.											
3/9	614	818	597	221	27	75	1.00	3,7	10,2	9.10	90	
3/9	Diet: 201 grams crackermeal, 10 grams lard, 10 grams butter, 2, Bland's pills daily											
3/15	756	869	600	269	31	87	0.99	4,4	14,8	9.00	97	*
3/20	801	843	531	312	37	95	1.00	4,7	11,4	8.50	99	*
3/27	848	848	517	331	39	100	0.89	5,6	8,2	8.40	101	*
4/3	943	865	519	346	40	109	0.88	6,2	18,8	8.30	104	*
4/9	984	878	536	342	39	112	0.85	6,6	11,4	8.30	106	*

* Slight anisocytosis of red blood cells.

Experimental history, see table 66-b.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

on crackermeal, lard and butter was the first anemia experiment done on this animal.

The control without Bland's pills and this experiment (table 70) with Bland's pills show practically identical anemia figures for red cell hematocrit and hemoglobin. The amount of red cell and hemoglobin regeneration is practically identical in the 5 weeks in the two experi-

ments. This again gives evidence that Blaud's pills are inert under these controlled conditions of experimental anemia and regeneration.

Table 71 shows a little more blood regeneration than usual but not enough to be able to attribute any of the reaction to the Blaud's pills. The red cell hematocrit reads 39 per cent at the end of 1 month and this is not a normal figure. The hemoglobin was low to start with

TABLE 72

*Blood regeneration—bread, milk and Blaud's pills—splenectomy. Dog 17-142.
Coach mongrel, female, age 4 to 5 months*

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
4/24	842	772	386	386	50	109	0.91	6,0	19,8	8.00	96
4/24	Diet: Bread and milk										
4/25	Bled 193 cc.										
4/26	Bled 193 cc.										
4/27	323	609	457	152	25	53	0.98	2,7	20,2	8.00	76
4/27	Diet: Bread, milk and 2 Blaud's pills daily										
5/2	390	600	390	210	35	65	0.90	3,6	18,4	7.80	76
5/9	760	835	451	384	46	91	0.83	5,5	13,6	7.90	106
5/16	837	790	411	379	48	106	0.80	6,6	7,2	7.70	103
5/23	997	906	453	453	50	110	0.75	7,3	6,4	7.70	117
5/30	890	832	416	416	50	107	0.70	7,7	11,4	8.00	104
6/6	950	863	440	423	49	110	0.73	7,5	11,6	7.70	112

No previous anemia experiments on this dog.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

and returns to this level in 4 weeks. The type reaction of this dog is well established by the list of other anemia experiments given in table 66-b.

Table 72 shows a reaction to the first anemia experiment which has been noted in other experiments. It is not the rule but is frequent enough so that we must always be on our guard in discussing the *first* anemia experiment on any given dog. There may be this remarkable

reserve which enables the dog to give an unusual blood regeneration even on a most unfavorable diet. The blood regeneration is complete in 3 to 4 weeks and thereafter is maintained for the next 3 weeks at

TABLE 73

*Blood regeneration—bread, milk and Bland's pills—splenectomy. Dog 17-37.
Bull mongrel, female, young adult*

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
3/26	927	850	459	391	46	109	0.80	6,8	9,4	10.60	80	
3/29	Bled 212 cc. and 212 cc. (refer to table 8)											
4/23	Diet: Bread and milk following fasting experiment of 3 weeks' duration											
4/23	540	772	502	270	35	70	0.66	5,3	7,8	6.90	112	*
4/30	578	802	521	281	35	72	0.67	5,4	11,6	8.20	98	
5/7	566	808	517	291	36	70	0.71	4,9	7,4	8.40	96	
5/14	662	808	517	291	36	82	0.68	6,0	6,8	8.40	96	
5/21	656	830	531	299	36	79	0.69	5,7	5,6	8.90	93	
5/28	566	833	553	283	34	68	0.68	5,0	7,0	8.30	100	
6/4	524	759	530	228	30	69	0.73	4,7	5,2	8.60	88	
6/11	528	754	529	226	30	70	0.78	4,5	14,6	8.60	88	
6/11	Diet: Bread, milk and 2 Bland's pills daily											
6/18	631	809	517	291	36	78	0.62	6,3	6,4	8.40	96	†
6/25	514	858	626	231	27	60	0.79	3,8	53,0	8.30	103	†
7/2	439	708	517	191	27	62	0.79	3,9	18,2	8.40	84	†
7/9	537	790	521	268	34	68	0.76	4,5	13,0	8.60	92	†
7/16	648	762	480	282	37	85	0.77	5,5	9,3	8.40	91	†

* Red cells are small and much fragmented.

† Red cells are large and fairly uniform in size.

Experimental history, see table 8-b.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

the normal level. Refer to tables 74 and 75 for meat diet controls with no previous bleeding. The fact that the dog was very young (4 to 5 months) and was without a spleen is thought to be without influence on this general reaction noted in other dogs (adult and non-

splencetomized). We have no reason to suspect that the Blaud's pills were concerned in this reaction which has been noted in control experiments on the same diet without the Blaud's pills.

The second splenectomy experiment (table 73) shows a negative reaction with bread and milk alone as well as with bread and milk plus Blaud's pills. There are peculiar fluctuations during certain weeks

TABLE 74

Blood regeneration—meat and Blaud's pills. Dog 17-191. Bull mongrel, male, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
4/24	1352	1206	567	640	53	112	0.71	7,9	13.4	9.50	127
4/24	Diet: Bread and milk										
4/25	Bled 301 cc.										
4/26	Bled 301 cc.										
4/27	382	780	600	187	24	49	0.79	3,1	17,6	9.30	84
4/27	Diet: Meat and 2 Blaud's pills daily										
5/2	536	800	543	264	33	67	0.91	3,7	17,8	9.50	84
5/9	1030	1064	585	479	45	97	0.90	5,4	8,2	9.50	112
5/16	1282	1256	653	604	48	102	0.73	7,0	11,8	9.80	128
5/23	1530	1377	647	730	53	111	0.66	8,4	8,8	9.70	142
5/30	1571	1366	615	752	55	115	0.65	8,8	18,6	10.00	136
6/6	1510	1314	591	419	55	115	0.66	8,7	7,2	9.60	137

No previous anemia experiments on this dog.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

in both these periods and we believe these ups and downs are referable to the splenectomy. It is significant that the hemoglobin and red cell hematocrit changes are not associated with any constant change in plasma volume. The red count fluctuates with the pigment curve and we must assume periodic constructive or destructive waves influencing the red cells in the blood stream. From data already published (1) dealing with bile excretion in splenectomized dogs with simple

anemia we may suspect that blood destruction may be in part responsible for these irregularities in the level of the curves of pigment volume, red cell and hemoglobin values. The color index shows no change. There is a note to the effect that the red cells are larger and more uniform in size during the period of iron feeding. A single observation of this nature is of interest but does not call for discussion at this time.

TABLE 75

Blood regeneration—meat and Bland's pills—splenectomy. Dog 17-163. Bull mongrel, male, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BY GOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
4/24	918	918	450	468	51	100	0.75	6,7	14,4	9.00	102
4/24	Diet: Bread and milk										
4/25	Bled 229 cc.										
4/26	Bled 229 cc.										
4/27	340	679	509	170	25	50	1.00	2,5	17,6	8.90	76
4/27	Diet: Meat and 2 Bland's pills daily										
5/2	538	803	514	289	36	67	0.90	3,7	19,2	9.00	89
5/9	790	909	509	400	44	87	0.78	5,6	19,6	8.90	102
5/16	924	880	475	405	46	105	0.78	6,7	8,2	8.90	98
5/23	1140	1096	559	538	49	104	0.75	6,9	12,4	8.80	124
5/30	907	863	423	440	51	105	0.63	8,3	9,2	9.00	96
6/6	1080	1002	491	520	51	106	0.65	8,2	13,8	8.60	117

No previous anemia experiments on this dog.

Experimental history, see table 38-b.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

The two meat feeding experiments (tables 74 and 75) give the expected reaction on this diet. The splenectomy dog reacts the same as does the normal dog. Sufficient meat is given to maintain a constant body weight. Both these dogs had not been used previously for anemia experiments; refer to table 72 which illustrates the reserve capacity sometimes exhibited by such dogs. We have no reason to suppose that the Bland's pills were in any way concerned in this reaction.

Hemoglobin experiments.—Table 76 gives an experiment of much interest and we are able to submit the control experiment (table 16). This same dog a few months previously was made anemic and placed upon a sugar diet. After a period of 4 weeks the level of pigment volume, hemoglobin and red cell hematocrit was practically the same as that

TABLE 76

Blood regeneration—hemoglobin and sugar—metabolism. Dog 17-28. Bull mongrel, female, young adult

DATE, 1917	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
5/7	1895	1709	769	940	55	111	0.66	8,4	13,2	12.50	136	
5/7	Diet: Mixed food											
5/8	Bled 427 cc.											
5/9	Bled 427 cc.											
5/11	594	914	640	274	30	65	0.76	4,3	22,0	12.20	76	
5/11	Diet: 50 grams cane sugar, 25 grams glucose, 10 grams washed red blood cells											
5/14	558	979	676	303	31	57	0.75	3,8	11,2	11.50	85	
5/21	676	1055	665	390	37	64	0.70	4,6	6,8	10.80	98	*
5/28	856	1141	673	468	41	75	0.71	5,3	9,6	10.10	113	*
6/4	887	1137	603	534	47	78	0.58	6,7	9,6	9.50	119	*
6/11	920	1180	672	508	43	78	0.59	6,6	8,0	8.90	133	*
6/18	1085	1277	664	612	48	85	0.50	8,5	10,0	8.30	154	*

* Marked fragmentation of red cells.

400 cc. water given by stomach tube daily.

Experimental history, see table 6-b; see table 16 for control.

Blood volume with dry oxalate. Hemoglobin by Sahli tubes.

observed immediately after the bleeding. The total blood regeneration then in the control experiment was zero. There was a trifling gain in the red count.

Under identical conditions on a sugar diet plus 10 grams washed, dried red blood cells we see a very different reaction (table 76). The

TABLE 76-A

Total urinary nitrogen—hemoglobin and sugar. Dog 17-28

DATE, 1917	TOTAL NITROGEN 24 HOURS URINE	URINE TOTAL 24 HOURS	WEIGHT	REMARKS
	<i>grams</i>	<i>cc.</i>	<i>pounds</i>	
5/11	3.55	490	26.4	0 feces
5/12	3.19	480	25.8	Solid black stool
5/13		361	25.5	Solid black stool
5/14	3.50	386	25.3	0 feces
5/15	2.52	485	25.0	Slight diarrhea
5/16	2.58	358	24.7	Solid feces
5/17	2.38	390	24.5	0 feces
5/18	2.35	480	24.4	Trace of feces
5/19	2.30	403	24.1	Slight diarrhea
5/20	2.46	410	24.0	0 feces
5/21	2.46	420	23.8	0 feces
5/22	2.63	465	23.4	0 feces
5/23	2.41	383	23.4	0 feces
5/24	2.94	400	23.0	Diarrhea +
5/25	2.74	376	22.7	Diarrhea +
5/26	2.66	412	22.6	0 feces
5/27	2.52	395	22.4	0 feces
5/28	2.35	424	22.2	0 feces
5/29	2.46	370	21.9	0 feces
5/30	2.63	410	21.8	0 feces
5/31	2.13	510	21.6	0 feces
6/1	2.32	405	21.4	0 feces
6/2	2.18	405	21.4	0 feces
6/3	2.30	425	21.1	0 feces
6/4	2.24	426	20.8	
6/5	2.44	425	20.8	0 feces
6/6	3.00	417	20.5	Formed feces
6/7	2.24	373	20.2	Soft feces
6/8	2.46	410	20.0	Solid feces
6/9	2.58	419	19.9	0 feces
6/10	2.60	425	19.8	0 feces
6/11	2.63	407	19.5	0 feces
6/12	2.83	405	19.5	0 feces
6/13	2.83	375	19.4	0 feces
6/14	2.88	490	19.2	0 feces
6/15	3.00	480	18.9	0 feces
6/16	3.28	411	18.6	Solid feces
6/17	3.42	395	18.4	0 feces
6/18	3.02	400	18.3	

initial anemia level in the two experiments is practically identical, also the body weight, normal initial blood pigment, etc. The only point in which these two experiments differ lies in the 10 grams of red cells added to the sugar diet. At the end of 4 weeks the control shows a gain of zero in pigment substance but the red cell feeding gives a substantial gain of 13 per cent hemoglobin, 17 per cent red cell hematocrit and 300 units pigment volume. There is a gain of 2,400,000 in the red cell count. The subsequent 2 weeks show a distinct gain over the level just noted. This is in notable contrast to the expected reaction on a sugar diet.

The urinary nitrogen and fluid excretion figures are given (table 76-a) and show the expected values. During the last week of the experiment there is a distinct rise in the nitrogen output. We have come to look upon this as an early sign of intoxication which if not heeded may be followed by severe clinical disturbances and death. This dog promptly recovered when placed on a mixed diet.

It is to be noted that *whole red cells* were used in this experiment, that is, hemoglobin plus red cell stroma. In the following experiments hemoglobin alone was used. We might point out the difference in the color index observed in these two conditions but feel that the data are not sufficient to establish this very interesting point. We are gradually collecting data which indicate the conditions most favorable for stroma production and these experiments will be presented at another time.

The next hemoglobin experiment (table 77) is not very convincing as we do not have a suitable control of the bread and milk factors in this dog. Moreover this is the first anemia experiment on this dog and under such circumstances this reaction is often atypical, as has been noted before. We may say that the blood regeneration due to the bread and milk plus hemoglobin might be identical with a reaction on bread and milk alone. We cannot point with certainty to any difference which can be attributed to the hemoglobin. This experiment is unlike the others of this group which give positive evidence that hemoglobin does influence the curve of blood regeneration. Finally, we must conclude that this experiment does not give any evidence against the value of hemoglobin but it also gives no positive support to the other experiments.

Table 78 presents a long experiment in which hemoglobin is given by mouth during one period and by intravenous injection during a subsequent period. We feel that there is good evidence that hemo-

globin did influence favorably the blood regeneration. The anemia level produced by three bleedings was slightly below the average. The first period of sugar feeding plus 10 grams of hemoglobin by mouth shows a notable gain during 3 weeks. The hemoglobin rises 50 per cent and the pigment volume from 433 to 810. There is a correspond-

TABLE 77

Blood regeneration—powdered hemoglobin and bread and milk. Dog 18-124. Terrier mongrel, female, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.
5/16		515	273	234	45.5					5.80	89
5/26	Diet: Bread and milk										
5/27	488	548	302	241	43.8	89	0.53	8,4	10,4	6.10	90
5/28	Bled 137 cc.										
5/29	Bled 137 cc. No distress										
5/29	Diet: 125 grams bread (ground and dried), 300 cc. milk, 5 grams hemo- globin*										
5/31	207	398	308	87	21.8	52	0.59	4,4	12,8	5.70	70
6/5	370	481	348	129	26.9	77	0.80	4,8	11,2	5.75	84
6/14	376	522	357	160	30.6	72	0.61	5,9	8,6	5.35	98
6/21	421	513	325	183	35.7	82	0.60	6,8	7,8	5.20	99
6/26	342	417	266	145	34.7	82	0.66	6,2	7,2	5.25	79

* Hemoglobin: Defibrinated blood centrifuged, cells washed twice with N/1 salt solution, 2 volumes distilled water added, allowed to lake over night, centrifugalized, stroma removed, hemoglobin dried and powdered.

No previous anemia experiments on this dog.

ing rise in the red cell hematocrit and red cell count. On sugar alone we recall that the gain in these pigment factors is only trifling, perhaps 10 per cent in hemoglobin and corresponding amounts in the other readings. We must hold the hemoglobin responsible at least for a part of this favorable reaction.

TABLE 78

*Blood regeneration—sugar and hemoglobin—crackermeal, milk and hemoglobin.
Dog 17-157. Coach mongrel, female, young adult*

DATE, 1918	PIGMENT VOLUME = Hb. PERCENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PLR KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
8/28	1366	1027	518	504	49.0	133	1.00	6,7	14,4	10.10	102	*Slight
8/28	Diet: Crackermeal and milk											
8/29	Bled 257 cc.											
8/30	Bled 257 cc.											
8/31	565	777	560	215	27.6	73				9.65	81	
8/31	Bled 194 cc.											
9/3	433	731	544	179	24.5	59	0.98	3,0	13,8	9.25	79	*Poik.+
9/10	Diet: 75 grams sugar, 25 grams dextrose, 10 grams hemoglobin† by stomach tube											
9/10	646	848	590	249	29.4	76	0.84	4,5	9,4	8.40	100	*Poik.+ Vomited
9/16	750	896	593	294	32.8	84	0.82	5,1	6,8	8.00	113	* Poik.+
9/25	810	788	492	293	37.2	102	0.86	5,9	6,6	7.25	109	* Poik.+
9/25	Diet: 100 grams sugar, 30 cc. hemoglobin intravenously‡											
9/30	625	742	463	271	36.5	84	0.70	6,0	6,2	6.95	106	* Poik.+
9/30	Diet: 200 grams crackermeal, 500 cc. milk, and hemoglobin intravenously‡											
10/11	662	752	465	280	37.2	88	0.76	5,8	13,6	7.55	100	
10/16	805	830	502	322	38.8	97	0.73	6,3	12,8	7.55	110	* Slight
10/16	Diet: 200 grams crackermeal, 500 cc. milk											
10/23	722	902	570	322	35.8	80	0.67	6,0	16,0	8.00	112	* Poik.+

* Poikilocytosis of red blood cells.

† Blood centrifugalized, washed once with salt solution; 2 volumes of distilled water added to washed, packed red blood cells. Allowed to stand 24 hours. Centrifugalized and stroma removed. Dried and powdered.

‡ For injection into the vein: With aseptic technique blood is centrifugalized, washed once with salt solution; 20 cc. distilled water added to 10 cc. washed, packed red blood cells. Allowed to stand 4 hours, added 2.5 cc. of 10 percent salt solution. Centrifugalized and stroma removed. Total amount injected intravenously daily.

Experimental history, see table 66-b.

When the sugar diet is continued but the hemoglobin is given intravenously, we note a fall in hemoglobin and pigment volume but no change in the red cell hematocrit, red cell count and plasma volume. We have no good explanation to fit these observed facts.

TABLE 79

Blood regeneration—hemoglobin injection intravenously. Dog 17–27. Bull mongrel, female, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
8/9	1914	1454	718	727	50.0	133	0.75	8,7	12,0	16.15	90	
8/9	Diet: Bread and milk											
8/12	Bled 364 cc. No distress											
8/13	Bled 364 cc. No distress											
8/15	749	1040	732	297	28.6	72				15.55	67	
8/15	Bled 260 cc.											
8/17	817	1034	718	311	30.1	79	0.95	4,1	24,6	15.45	67	* Poik. ++
8/17	Diet: Hemoglobin injection intravenously; † 75 grams sugar, 25 grams dextrose, 200 cc. water daily by stomach tube											
8/23	908	1080	710	370	34.3	84	0.76	5,5	14,8	14.55	74	
8/30	1155	1100	647	442	40.2	105	0.75	7,0	5,6	13.75	80	* Poik. ++
9/2	Accidental death											

* Poikilocytosis of red blood cells.

† Hemoglobin: Blood drawn from normal dog with aseptic precautions. Centrifugalized, washed once with salt solution; 20 cc. distilled water added to 10 cc. washed, packed red blood cells. Allowed to stand 4 hours, added 2.5 cc. of 10 per cent salt solution. Centrifugalized and stroma removed. Total amount injected intravenously daily.

Experimental history, see table 15-b; see sugar control, table 15.

The 2 weeks following on a crackermeal and milk diet plus hemoglobin intravenously show a slight gain in pigment substance. Even this slight gain may have some significance when we recall that it

occurred following a long period of limited diet intake. Hemoglobin regeneration under such unfavorable circumstances is very difficult and becomes increasingly difficult as the limited diet periods are extended.

Table 79 gives an experiment which was unfortunately terminated at the end of 2 weeks by an accident. We are able to refer to a control reaction on sugar feeding alone (table 15). This table shows a slight gain in hemoglobin, red cell hematocrit and pigment volume during the first week on the sugar diet. The second week shows no gain. Compare with this reaction the figures in table 79 which show a gain in the first week which may be compared with the control but the second week instead of the stationary level in the control shows a distinct gain in red cells, pigment volume, hemoglobin, etc. We feel that a part of this gain is to be explained by the hemoglobin injections.

Table 80 illustrates another type of experiment in which the hemoglobin injections are given under conditions very unfavorable for blood regeneration. There is a 3-week period of sugar feeding during which time there is zero gain in pigment substance. There is a slight but distinct gain following 5 days of hemoglobin injection and further slight gains on a crackermeal and milk diet with daily hemoglobin injections. Some of the subsequent gains in hemoglobin, red cell hematocrit and red cells may be due in part to the hemoglobin injections which in all probability cannot be at once utilized. The crackermeal and milk alone or with yeast can account for very little blood regeneration when given subsequent to a long period of sugar feeding. The evidence for the favorable influence of hemoglobin injections is not as strong as in some of the other experiments which have the complete control periods.

The last hemoglobin experiment (table 81) is to be compared with table 76. The hemoglobin in this instance is given intraperitoneally so that the absorption might be rapid and the elimination of slight amounts through the urine be obviated. The influence of a different set of phagocytic cells might well be a factor but the gross results are much the same as regards blood regeneration.

Three weeks of sugar feeding plus hemoglobin injection give an amount of blood regeneration which cannot be explained as due to the sugar feeding. We note a rise of hemoglobin from 72 to 120 per cent and red cell hematocrit from 27 to 45 per cent. The control sugar diet figures would show only trifling gains. Subsequent weeks on a crackermeal and milk diet do not show much gain except in the red count. The final period of mixed diet as usual brings the dog back to a high normal figure.

TABLE 80

Blood regeneration—hemoglobin intravenously. Dog 16-160. Bull mongrel, female, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm.	cc.	
8/28	1835	1103	456	654	55.2	166	1.10	7,6	9,6	10.15	109	
8/28	Diet: Bread and milk											
8/29	Bled 276 cc.											
8/30	Bled 276 cc.											
9/1	648	771	524	243	31.5	84				9.55	81	*
9/1	Bled 193 cc.											
9/3	448	696	513	177	25.5	64	0.80	4,0	15,4	9.50	73	* Poik.++
9/3- 9/26	Diet: 75 grams cane sugar, 25 grams dextrose, 200 cc. water by stomach tube (table 18)											
9/25	429	664	474	182	27.4	64	0.61	5,2	9,6	7.35	90	* Poik.++
9/26	Diet: Hemoglobin intravenously, ‡ 30 cc., and 100 grams sugar by stomach tube											
9/30	437	618	421	194	31.4	71	0.68	5,2	12,4	7.15	86	* Poik.+++
9/30	Diet: Hemoglobin intravenously, ‡ 30 cc., and 200 grams crackermeal, 500 cc. milk											
10/11	506	712	477	227	31.9	71	0.58	6,1	11,0	7.75	92	* Poik.++
10/16	586	740	495	237	32.0	79	0.65	6,1	5,0	7.80	95	* Poik.+++
10/16	Diet: Hemoglobin injection discontinued; 200 grams crackermeal, 500 cc. milk											
10/23	670	826	536	281	34.0	81	0.65	6,2	8,4	8.05	103	* Poik.+++
10/23	Diet: 1 gram dried brewer's yeast, 200 grams crackermeal, 500 cc. milk.											
10/30	590	808	536	264	32.7	73	0.47	7,7	15,2	8.30	97	* Poik.+++
11/6	720	868	547	217	36.5	83	0.66	6,3	8,6	8.20	106	* Poik.+
11/12	784	883	532	342	38.7	89	0.50	8,9	7,2	8.25	107	* Poik.+
11/13	Diet: Mixed diet											
11/22	726	853	503	341	40.0	85	0.42	10,2	13,6	8.80	97	* Poik.+
11/29	1122	1020	547	463	45.4	110	0.69	8,0	19,0	9.25	105	Excellent condition

* Poikilocytosis of red blood cells.

† Shadow cells.

‡ Hemoglobin: Blood taken with aseptic precautions, centrifugalized, washed once with salt solution; 20 cc. of distilled water added to 10 cc. washed, packed red blood cells. Allowed to stand 4 hours, added 2.5 cc. of 10 per cent salt solution. Centrifugalized and stroma removed. Total amount injected intravenously daily.

Experimental history, see table 18-b.

TABLE 81

Blood regeneration—hemoglobin intraperitoneally. Dog 17-23. Bull mongrel, female, young adult

DATE, 1918	PIGMENT VOLUME = Hb. PER CENT TIMES BLOOD VOLUME	BLOOD VOLUME	PLASMA VOLUME	R. B. C. VOLUME	R. B. C. HEMATOCRIT	Hb.	COLOR INDEX	R. B. C. MILLION	W. B. C.	WEIGHT	BLOOD PER KILOGRAM	REMARKS
		cc.	cc.	cc.	per cent	per cent				kgm	cc.	
8/9	2417	1580	680	887	56.1	153	0.84	9,1	14,2	17.00	93	
8/9	Diet: Bread and milk											
8/12	Bled 395 cc. No distress											
8/13	Bled 395 cc. Diet: Crackermeal and milk											
8/15	1015	1142	750	387	33.8	89				16.30	71	
8/15	Bled 286 cc.											
8/17	756	1050	758	281	26.8	72	0.97	3,7	23,6	15.90	66	* Poik. +
8/19	Hemoglobin† (intraperitoneal injection). Diet: 75 grams sugar, 25 grams dextrose, water											
8/23	794	1030	708	310	30.1	77	0.69	5,6	21,4	15.40	67	* Poik.
8/30	1133	1193	685	430	36.0	95	0.76	6,2	12,6	14.55	82	* Poik. +
9/4	Hemoglobin† (intraperitoneal injection). Diet: 100 grams sugar in 500 cc. water											
9/6	1670	1385	755	608	45.0	120	0.83	7,2	11,0	13.40	103	* Poik. ++
9/6	Hemoglobin discontinued. Diet: 200 grams crackermeal, 500 cc. milk kaolin											
9/13	1550	1450	876	566	39.2	107	0.68	7,9	9,8	14.00	103	* Poik. * Poik.
9/19	1433	1310	752	545	41.6	109	0.71	7,7	12,2	13.90	94	
9/27	1253	1130	658	468	41.4	111	0.71	7,8	10,6	14.30	79	
10/9	1635	1363	668	682	50.5	120	0.77	7,8	12,2	14.20	96	
10/18	1836	1386	680	694	50.0	132	0.66	10,1	9,0	14.45	96	
10/25	1550	1352	704	636	47.0	115	0.64	9,0	10,8	14.60	93	
10/25	Diet: 200 grams crackermeal, 500 cc. milk, 1 gram dried, powdered brewer's yeast, kaolin											
10/31	1415	1400	750	638	45.4	101	0.65	7,8	11,8	15.00	93	
10/31	Diet: Mixed diet											
11/7	1975	1555	710	829	53.3	127	0.74	8,6	10,8	15.10	103	
11/13	2000	1600	743	840	52.5	125	0.63	9,9	20,2	15.05	106	

* Poikilocytosis of red blood cells.

† Hemoglobin: 10 cc. sterile, washed, packed red blood cells and 20 cc. distilled water. Centrifugalized and stroma removed. Intraperitoneal injection.

Experimental history, see table 6-b; see table 76 for hemoglobin feeding.

SUMMARY

Blaud's pills are inert when added to various diets which do or do not favor rapid blood regeneration. We may not assume without positive proof that inorganic iron is of value in the treatment of secondary anemia.

Splenectomy may or may not modify this blood regeneration reaction. Limited diets following anemia periods associated with splenectomy may be the cause of fluctuations in the normal expected curve of blood regeneration.

Hemoglobin (by mouth, intravenously or intraperitoneally) exerts a distinctly favorable influence upon subsequent blood regeneration.

BIBLIOGRAPHY

- (1) HOOPER AND WHIPPLE: This Journal, 1917, xliii, 275.

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